Diplomarbeit

Expression and phosphorylation of Ca^{2+} handling proteins in murine hearts with a ryanodine receptor mutation after pressure-induced hypertrophy

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Zusammenfassung

HINTERGRUND: Fehlfunktionen des kardialen Ryanodin-Rezeptors (RyR2) führen zu einer spontanen Kalziumfreisetzung aus dem sarkoplasmatischen Retikulum (SR). Dies hat Arrhythmien und eine verminderte Kontraktionsfähigkeit des Herzen zur Folge. Diese Fehlfunktionen können entweder kongenital, wie bei mit katecholaminerger polymorpher ventrikulärer Tachykardie (CPVT) assoziierten RyR2 Mutationen oder aber im Rahmen einer Herzinsuffizienz erworben sein.

ZIEL: Die vorliegende Arbeit untersucht die Fragestellung, ob eine erhöhte spontane Ca^{2+}-Freisetzung aus dem SR aufgrund einer RyR2-Mutation und Druckbelastung die Expression und Phosphorylierung ausgewählter kardialer Kalzium (Ca^{2+})-regulierender Proteine in Mäuseherzzellen verändert.

METHODEN: Die Experimente wurden an Mäuseherzen mit einer CPVT-assoziierten $RyR2^{R4496C+/-}$ (R4496C)-Mutation eine Woche nach einer Drucklast-Induktion durch minimal-invasive transverse Aortenkonstriktion (TAC) oder Scheinoperation (Sham) durchgeführt. Die Quantifizierung ausgewählter Ca^{2+}-regulierender Proteine, einschließlich Phospholamban (PLB), SR Ca^{2+}-ATPase 2a (SERCA2a) und Calsequestrin 2 (CASQ2) erfolgte mittels Western Blot. Phosphorylierungsstellen-spezifische Antikörper wurden zur Evaluierung des Phosphorylierungsstatus von RyR2 und PLB durch die Proteinkinase A (PKA), an RyR2-Ser2808 und PLB-Ser16, sowie die Ca^{2+}/Kalmodulin-abhängige Proteinkinase II (CaMKII), an RyR2-Ser2814 und PLB-Thr17, verwendet.

ERGEBNISSE: Während die Expression von CASQ2 und PLB innerhalb der ersten Woche unverändert blieb, zeigte sich eine deutliche Reduktion der SERCA2a-Expression in R4496C-TAC- und WT-TAC-, verglichen mit den WT-Sham- Mäuseherzen. Die Phosphorylierung von RyR2 war sowohl an der PKA-abhängigen Stelle (Ser2808) als auch an der CaMKII-abhängigen Stelle (Ser2814) unverändert. Die PKA hatte weiters keinen Effekt auf PLB. An der CaMKII-abhängigen PLB-Stelle (PLB-Thr17) zeigte sich jedoch eine signifikante Reduktion der Phosphorylierung in den R4496C-Mäuseherzen, sowohl in der TAC- als auch in der Sham-Gruppe, verglichen mit den WT-Sham Herzen.

SCHLUSSFOLGERUNG: Eine verminderte SERCA2a-Expression und PLB-Phosphorylierung lassen auf eine verminderte SERCA2a-Aktivität in den R4496C-TAC-Mäuseherzen schließen. Unsere Daten zeigen eine gestörte SR-Ca^{2+}-

Wiederaufnahme und daher einen verminderten SR Ca^{2+}-Gehalt. Dies führt zur Kontraktionsschwäche in den R4496C-Mäuseherzen nach Druckbelastung.

Abstract

BACKGROUND: Cardiac ryanodine receptor (RyR2) dysfunction causes spontaneous sarcoplasmic reticulum (SR) calcium (Ca^{2+}) release, leading to arrhythmias and reduced contractile function. RyR2 dysfunction can be acquired, as in heart failure (HF), or inherited by RyR2 mutations, as in catecholaminergic polymorphic ventricular tachycardia (CPVT).

AIM: The aim of the present study was to elucidate whether an increased SR Ca^{2+} leak due to a RyR2 mutation alters the expression of cardiac Ca^{2+} handling proteins and their phosphorylation status in murine cardiomyocytes during pressure overload-induced hypertrophy.

METHODS: We investigated mice harboring a CPVT-associated $RyR2^{R4496C+/-}$ (R4496C) mutation and their WT littermates one week after pressure-induced overload induced by minimally invasive transverse aortic constriction (TAC). Mice undergoing the surgery without aortic ligation served as controls (Sham). Quantification of Ca^{2+} handling proteins, including phospholamban (PLB), SR-Ca^{2+}-ATPase 2a (SERCA2a) and calsequestrin (CASQ2), was performed by Western blotting. Phospho-site-specific antibodies were used for evaluation of the phosphorylation status of RyR2 and PLB by protein kinase A (PKA) (RyR2 at Ser2808 and PLB at Ser16, respectively) as well as Ca^{2+}/calmodulin-dependent kinase II (CaMKII) (RyR2 at Ser2814 and PLB at Thr17, respectively).

RESULTS: We observed unchanged expression of CASQ2 and PLB, however, SERCA2a expression was significantly reduced in both WT-TAC and R4496C-TAC mice in respect with WT-Sham. RyR2 phosphorylation status at PKA- and CaMKII-dependent sites, Ser2808 and Ser2814, respectively, was unchanged. While PKA had also no effect on the phosphorylation of PLB, the phosphorylation level at the CaMKII-dependent site (PLB-Thr17) was significantly reduced in both R4496C-Sham and R4496C-TAC hearts, compared to WT-Sham.

CONCLUSION: Reduced SERCA2a expression and phosphorylation of PLB imply reduced SERCA2a activity in R4496C-TAC mice. Our data suggest an impaired SR Ca^{2+} reuptake and, thus, reduced SR Ca^{2+} content underlying contractile dysfunction in R4496C mice imposed to pressure overload.

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Glossary and abbreviations

$[Ca^{2+}]_i$	Intracellular calcium concentration (cytosolic calcium)
$[Ca^{2+}]_{SR}$	Sarcoplasmic reticulum calcium content/concentration
ACE	Angiotensin converting enzyme
ATP	Adenosine triphosphate
BCA	Bichinchoninic acid
BSA	Bovine serum albumin
Ca^{2+}	Calcium ion
CaM	Calmodulin
CaMKII	Calcium/calmodulin-dependent kinase II
cAMP	Cyclic adenosine monophosphate
CASQ	Calsequestrin
CICR	Calcium-induced Calcium release
Cn	Calcineurin
CPVT	Catecholaminergic polymorphic ventricular tachycardia
Cu^{2+}	Copper
$CuSO_4$	Copper sulfate
CWS	Chemiluminescence working solution
DAD	Delayed afterdepolarization
ECC	Excitation-contraction coupling
ECG	Electrocardiogram
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethyleneglycoltetraacetic acid
ETC	Excitation-transcription coupling
FKBP12.6	FK506 binding protein 12.6
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
HB	Homogenization buffer
HDAC	Histone deacetylase
HF	Heart failure
I_{ca}	Inward Ca^{2+} current
ICD	Implantable cardiac defibrillator
IP3	Inositol 1,4,5-triphosphate
JN	Junctin
LVH	Left ventricular hypertrophy
MAPK	Mitogen activated protein kinase
MI	Myocardial infarction
Na_2CO_3	Sodium carbonate
NA_3VO_4	Sodium orthovanadate
NaCl	Sodium chloride
NaF	Sodium fluoride
$NaHCO_3$	Sodium bicarbonate
NaOH	Sodium hydroxide
NCX	Sodium/calcium exchanger
NFAT	Nuclear factor of activated T-cells
NP40	Tergitol-type NP-40 (nonyl phenoxypolyethoxylethanol)
NYHA	New York Heart Association
PAD	Primary antibody dilution
PDE4D3	Phosphodiesterase 4D3

PKA	Protein kinase A
PKC	Protein kinase C
PLB	Phospholamban
PMSF	Phenylmethylsulfonyl fluoride
PP1	Protein phosphatase 1
PP2	Protein phosphatase 2
RPM	Rounds per minute
RT	Room temperature
RyR2	Ryanodine receptor 2
SAD	Secondary antibody dilution
Ser16	PLB residue serine 16
Ser2808	RyR2 residue serine 2808
Ser2814	RyR2 residue serine 2814
SERCA	SR-Ca^{2+} ATPase pump
SOICR	Store overload induced calcium release
SR	Sarcoplasmic reticulum
SWR	Standard working reagent
TAC	Transverse aortic constriction
Thr17	PLB residue threonine 17
TnC	Troponin C
TRD	Triadin
Tris HCL	Tris(hydroxymethyl)aminomethane hydrochloride
VT	Ventricular tachyarrhythmias
WT	Wild-type

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1. Introduction

1.1 Calcium cycling

1.1.1 Calcium

In cardiomyocytes, calcium (Ca^{2+}) plays an important role in excitation-contraction coupling (ECC), energy production (through mitochondrial ATP production), transcriptional gene regulation as well as cell death by apoptosis and necrosis.
Although these diverse Ca^{2+}-dependent processes occur simultaneously within the cell, it can distinguish, for example, between Ca^{2+} signals involved in ECC and those targeting gene expression via local Ca^{2+} signaling.
Specialized proteins, including pumps and ion channels, help the cell to maintain (within a certain range) a constant cytosolic Ca^{2+} concentration, a process termed Ca^{2+} homeostasis. Short term disturbances of the Ca^{2+} homeostasis result in mechanical and electrical dysfunction, while longer term adaptations involve altered protein expression, cell growth, and cell death (Bers 2008).

1.1.2 Sarcoplasmic reticulum

The sarcoplasmic reticulum (SR) is an 'intracellular, membrane bounded compartment not continuous with the sarcolemma' (Bers 2001). The SR is a major intracellular Ca^{2+} store, its main function is the sequestration and release of Ca^{2+} from and to the cytoplasm, respectively. The junctions of the SR with the sarcolemma are highly specialized, featuring type 2 ryanodine receptors (RyR2s), the main intracellular SR Ca^{2+} release proteins. The SR membrane contains mainly the SR Ca^{2+-}ATPase (SERCA2a), which takes up Ca^{2+} from the cytoplasm (Bers 2001).
Given the importance of the SR during contraction and relaxation, the SR Ca^{2+} content ($[Ca^{2+}]_{SR}$) is tightly regulated. A high $[Ca^{2+}]_{SR}$ enhances fractional SR Ca^{2+} release for a given inward Ca^{2+} current (I_{Ca}), due to a stimulatory effect of $[Ca^{2+}]_{SR}$ on RyR2 open probability. This mechanism can also cause an increased SR Ca^{2+} leak during diastole, resulting in an increased rate of Ca^{2+} sparks and Ca^{2+} waves

(a Ca^{2+} spark activating neighboring couplons and propagating over the whole cell), leading to aftercontractions and delayed afterdepolarizations, which can trigger arrhythmias.

Low $[Ca^{2+}]_{SR}$, as in HF, on the other hand, can contribute to the turn-off of SR Ca^{2+} release during ECC. The SR Ca^{2+} content can be raised by enhancing SR Ca^{2+} reuptake through SERCA2a, which is induced by β-adrenergic stimulation or phosphorylation of phospholamban (PLB), SERCA2a's main regulatory protein. At low $[Ca^{2+}]_{SR}$, I_{Ca} can fail to induce SR Ca^{2+} release, which allows more Ca^{2+} influx (due to lower local L-type Ca^{2+} channel inactivation) and less Ca^{2+} extrusion via the Na^+/Ca^{2+} exchanger (NCX), resulting in SR reloading (Bers 2002).

1.2 Excitation-Contraction Coupling

1.2.1 Calcium-induced calcium release

An action potential activates voltage-dependent L-type Ca^{2+} channels, resulting in a small Ca^{2+} influx, which triggers a quantitatively larger Ca^{2+} release from the SR, through the RyR2s. This process is termed 'calcium-induced calcium release' (CICR) and has been widely accepted to be the physiological mechanism of Ca^{2+} release in cardiac myocytes (Endo 2009). CICR is defined as 'Ca^{2+} release induced by Ca^{2+} alone, without the action of other activating processes' (Endo 2009). CICR increases cytosolic $[Ca^{2+}]$ from 100 nM to about 1μM. The released calcium binds to troponin C (TnC), thereby activating numerous processes resulting in contraction (Bers 2001). Contractile failure, as in HF, results from reduced SR Ca^{2+} content, with less Ca^{2+} available for CICR.

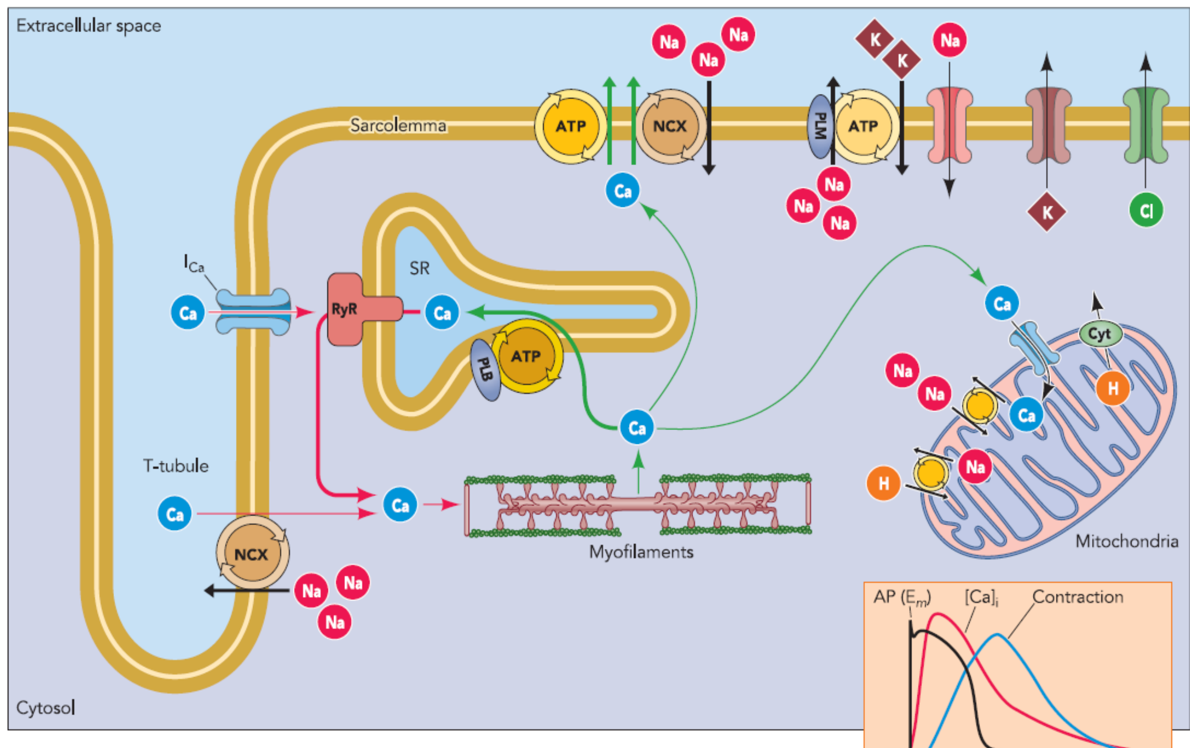


Figure 1: Ca^{2+} cycling in cardiac myocytes
Calcium enters the cell via the L-type Ca^{2+} channels located in the t-tubule, triggering a quantitatively larger Ca^{2+} release from the SR via RyR. $[Ca^{2+}]_i$ binds to troponin C (TnC), leading to myofilament contraction.
For relaxation to occur, $[Ca^{2+}]_i$ needs to decline for Ca^{2+} to dissociate from TnC. This process is mainly mediated by SERCA2a, pumping Ca^{2+} back into the SR; as well as NCX, which transports Ca^{2+} into the extracellular space. A small amount of Ca^{2+} is also pumped into mitochondria.
Ca^{2+} increase in the cytoplasm: red arrows, Ca^{2+} extrusion out of the cytoplasm: green arrows. Adopted from (Bers, Guo 2005)

1.2.2 Relaxation

For relaxation to occur, $[Ca^{2+}]_i$ must decline for Ca^{2+} to dissociate from the TnC binding sites. While the exact mechanisms remain controversial, depletion of SR Ca^{2+} as well as RyR2 inactivation or adaptation contribute to termination of CICR (Bers 2002).

Four Ca^{2+} transport mechanisms are involved in reducing cytosolic $[Ca^{2+}]$, including SERCA2a, NCX, sarcolemmal Ca^{2+}-ATPase and the mitochondrial Ca^{2+} uniporter (Bassani, Bassani & Bers 1992). These systems compete for the cytosolic Ca^{2+}. In rabbit and human ventricular myocytes, about 70% of the cytosolic Ca^{2+} is transported back into the SR by SERCA2a, while SERCA2a activity contributes about 92% in rat and murine myocytes. NCX adds 28% and 7% to cytosolic Ca^{2+} reduction, respectively. The remaining Ca^{2+} (about 1-2 %) is

sequestered from the cytosol by the sarcolemmal Ca^{2+}-ATPase and mitochondrial Ca^{2+}-uniporter (Bers 2002, Bassani, Bassani & Bers 1992). To maintain Ca^{2+} homeostasis, the amount of Ca^{2+} leaving the cell during relaxation must equal the amount of Ca^{2+} entry during contraction, for the cell not to gain or lose Ca^{2+} (Bers 2002).

1.3 Excitation-Transcription Coupling

Calcium further plays a crucial role in cardiac signal transduction by activating different kinases and phosphatases, regulating cardiac growth and function. This process of driving transcriptional responses is termed excitation-transcription coupling (ETC) (Seidler, Hasenfuss & Maier 2007).

Disturbances in calcium handling, like increased diastolic Ca^{2+}, directly activate a number of signaling proteins, such as Ca^{2+}/calmodulin-dependent kinase II (CaMKII), protein kinase C (PKC) and/or calcineurin. Calcineurin (Cn), a serine-threonine phosphatase, is activated by calmodulin (CaM), a Ca^{2+} sensor protein. Cn dephosphorylates NFAT (nuclear factor of activated T-cells) transcription factors, allowing nuclear localization and activation of a pro-hypertrophic gene program. Cn gene knockout mice as well as mice treated with calcineurin inhibitors are resistant to hypertrophy after aortic banding or isoproterenol infusions, while Cn overexpression causes massive hypertrophy in mice (Sussman et al. 1998, Seidler, Hasenfuss & Maier 2007).

CaMKII is activated by Ca^{2+}-calmodulin. It activates pro-hypertrophic pathways, mainly via histone deacetylases (HDACs). Importantly, HDAC nuclear export is regulated by Ca^{2+} release through inositol 1,4,5-trisphosphate (IP3) receptors, unrelated to overall cytosolic Ca^{2+} levels, further highlighting the importance of compartmentalized Ca^{2+} signaling (Seidler, Hasenfuss & Maier 2007).

ETC may also be involved in altered transcription of key Ca^{2+} transport and regulatory proteins, like SERCA2a, PLB or RyR2. Thus, this long term feedback loop could either feed back to restore normal cardiac myocyte function or it could contribute to exacerbation of hypertrophy or heart failure (Bers, Guo 2005).

1.4 Heart failure

Heart failure (HF) is a condition, in which the heart can no longer provide adequate blood flow to meet the body's demand. This results in typical clinical symptoms of HF like shortness of breath, reduced exercise tolerance, fatigue or fluid retention.
About half of the patients with HF symptoms suffer from systolic HF determined by contractile failure of the dilated heart, while the other half experience diastolic HF characterized by preserved ejection fraction. Patients with diastolic HF show almost normal contraction and non-dilated but hypertrophied hearts (Mudd, Kass 2008).

1.4.1 Epidemiology

Chronic heart failure is one of the leading causes of death in the industrialized world. Because the prevalence of HF increases dramatically with age, a significant rise in the number of patients can be seen in our aging population. At 40 years of age, the lifetime risk for developing HF for both men and women is 1 in 5, while at 80 years of age, the remaining lifetime risk for the development of new HF remains at 20%, even in the face of a much shorter life expectancy (Roger et al. 2011).
HF prevalence in European countries is about 0.3-2.4% in the general population with a rise in over 65 year olds to about 3.0-13.0% (Rickenbacher 2001). Although incidence is higher in men, greater live expectancy of women balances overall prevalence (McMurray, Stewart 2000).

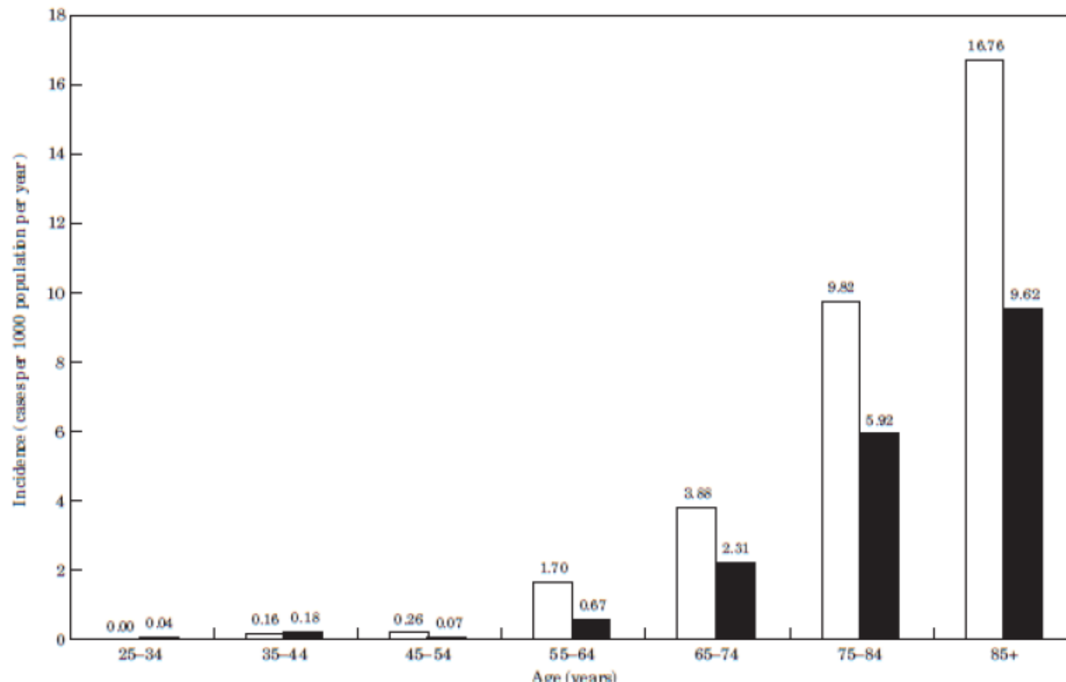


Figure 2: HF incidence

In a 20 month period, 220 patients with new diagnosis of HF were identified in a population of 151 000. Incidence increased steadily from 0.02/1000/year in patients aged 25-34 years to 11.6 in those aged 85 years or older and was higher in males (white bars) than in females (black bars). Criteria for the diagnosis of HF were adopted from recommendations by the Working Groups on Heart Failure of the European Society of Cardiology. Patients had to have appropriate symptoms (fluid retention, fatigue or shortness of breath) in the presence of an abnormality of cardiac structure and function (Cowie et al. 1999).

In Austria, 160.000 patients suffer from HF (Kozanli. et al. 2008) 35.000 of them being hospitalized each year (70% of them being older than 65 years) with an average duration of stay of 28 days. The estimated annual costs are about 340 million euro. Overall, the mortality is about 50% in 4 years after diagnosis, being 50% within one year for NYHA stages III and IV (Höllinger 2011).

1.4.2 HF classification

Among different classification criterias for staging HF in patients, the NYHA (New York Heart Association) functional classification system is the most common. According to the HF stage, a selected therapy is applied.

Table 1: NYHA heart failure classification and therapy (Erdmann 2011)

NYHA classification of HF		Therapy
Class I (asymptomatic HF)	no limitation of physical activity but echocardiographic alterations	lifestyle changes eventually ACE inhibitors
Class II (mild HF)	slight limitation of physical activity	ACE inhibitors; β-blockers and diuretics as needed
Class III (moderate HF)	marked limitation of physical activity	ACE inhibitors, β-blockers, diuretics and aldosterone antagonists
Class IV (severe HF)	no physical activity possible; symptoms of cardiac insufficiency at rest	ACE inhibitors, β-blockers, diuretics, aldosterone antagonists, low dose cardiac glycosides

1.4.3 Development of heart failure

There is a wide range of mechanisms and signaling pathways involved in the development of heart failure, with many of them overlapping. Generally, HF is characterized by a chronic hyperadrenergic state due to activated β-adrenergic receptors, leading to further activation of stress response protein kinases and phosphatases, such as protein kinase A (PKA), PKC, CaMKII, mitogen-activated kinases (MAPKs) and Cn. Their chronic activation leads to changes in cellular structure, function and overall regulation of the heart, referred to as cardiac remodeling (Mudd, Kass 2008, Lehnart, Maier & Hasenfuss 2009). Cardiac remodeling leads to the typical heart failure phenotype regardless of the initial cause (e.g., myocardial infarction, viral myocarditis, toxic cardiomyopathy, hypertension and mutations of calcium regulatory proteins).

About 50% of all deaths in HF patients are sudden and unexpected, mainly due to ventricular tachyarrhythmias (VT) (Grimm, Maisch 2002). Different aspects of cardiac remodeling and changes in Ca^{2+} handling contribute to the occurrence of delayed afterdepolarizations, being one possible cause of VT and sudden cardiac death.

1.5 Diastolic SR Ca^{2+} leak

RyR2 dysfunction, either acquired (such as in HF) or congenital (due to RyR2 mutations), leads to an increased spontaneous SR Ca^{2+} release via RyR2 ('RyR2 leak'). An increased SR Ca^{2+} load, a RyR2 dysfunction or a combination of both causes an increased diastolic SR Ca^{2+} release. This inappropriately timed spontaneous Ca^{2+} efflux from the SR activates Na^+/Ca^{2+} exchanger to operate in forward mode, leading to depolarization of the cardiomyocyte (Blayney, Lai 2009).

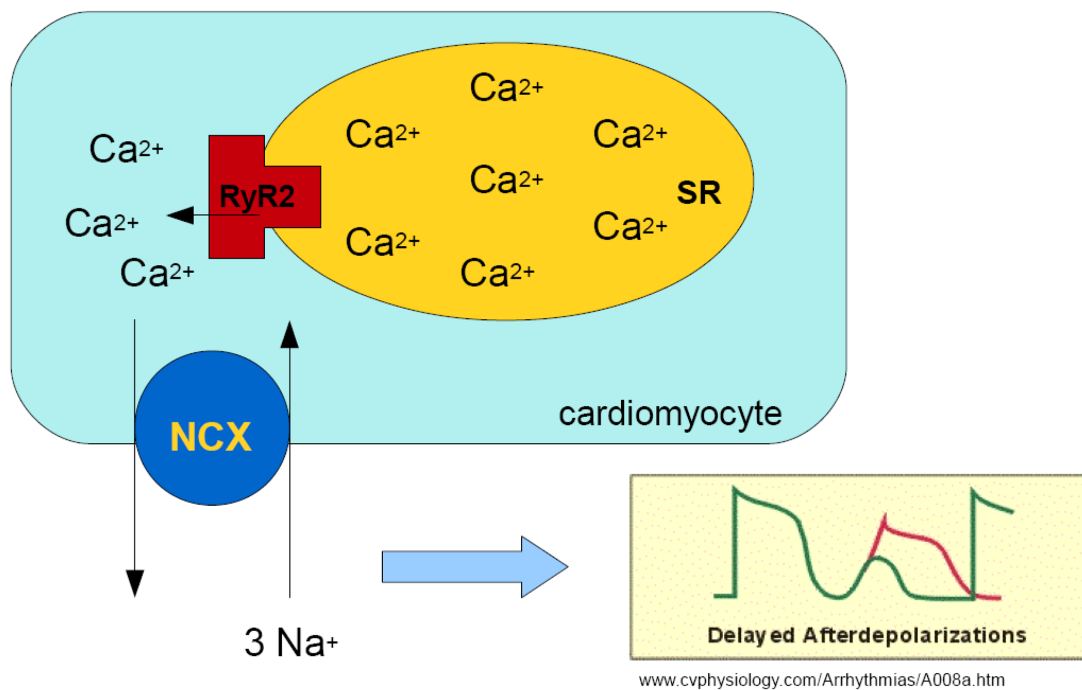


Figure 3: Diastolic SR Ca^{2+} release
Spontaneous SR Ca^{2+} release via dysfunctional RyR2 in HF or due to a congenital RyR2 mutation: The elevated diastolic [Ca^{2+}] concentration generates a transient inward current of 3 Na^+ in exchange for one Ca^{2+} through NCX, leading to membrane depolarization and generation of delayed afterdepolarizations (DADs).

In cardiac myocytes from failing hearts, increased SR Ca^{2+} release via the RyR2 as well as an upregulated NCX expression result in a larger transient inward current, which facilitates the development of delayed afterdepolarizations (DADs) (Pogwizd, Bers 2004).

RyR2 mutations or enhanced phosphorylation sensitize the channel to Ca^{2+} and the increased SR Ca^{2+} release results in decreased $[Ca^{2+}]_{SR}$ load. During β-adrenergic stimulation, however, increased SERCA2a activity and PLB

phosphorylation increase SR Ca^{2+} content, therefore enhancing SR Ca^{2+} release and facilitating the development of triggered arrhythmias (Blayney, Lai 2009).

1.6 Calcium regulatory proteins

Calcium homeostasis in cardiac myocytes is a very delicately balanced process, which is maintained by some key calcium handling proteins, including RyR2, SERCA2a together with PLB and CASQ2.

An increasing body of evidence suggests that alterations in Ca^{2+} homeostasis underlie defects in ECC, contributing to the pathophysiology of HF. HF is associated with cardiac remodeling, with many of the functional changes resulting from altered patterns of gene expression (Blayney, Lai 2009). Furthermore, numerous proteins are involved in the Ca^{2+} cycling process, such that alterations in expression, function or regulation of any single one or a combination of them may impair intracellular Ca^{2+} homeostasis, leading to the development of HF (Gregory, Kranias 2006).

A downregulation of SERCA2a together with an upregulation of NCX tends to extrude more Ca^{2+} from the cell, therefore reducing $[Ca^{2+}]_{SR}$. Another factor contributing to a reduction in $[Ca^{2+}]_{SR}$ is an enhanced diastolic SR Ca^{2+} release due to a higher RyR2 open probability. This leak results in a reduced contractile function as well as an enhanced propensity for arrhythmias (Bers 2006).

Furthermore, reduced Ca^{2+} transient amplitude and an increase in transient duration and decay time, largely due to reduced SERCA2a expression and activity, can be observed in failing cardiac myocytes.

Sustained elevation of $[Ca^{2+}]_i$ plays a key role in the activation of several pro-hypertrophic pathways and altered gene expression. Since PKA and CaMKII have dual roles in ECC and ETC, phosphorylating components of either pathway, changes in the expression of many ECC-associated proteins have been reported in animal models of HF and in failing human hearts (Pieske 2002, Mudd, Kass 2008, Blayney, Lai 2009, Hasenfuss, Duan 2010).

1.6.1 Ryanodine Receptor

RyRs are the main Ca^{2+} release channels, located in the membrane of the SR. They are responsible for Ca^{2+} release from the intracellular Ca^{2+} stores during ECC in both cardiac and skeletal muscle. RyRs were discovered when testing the plant alkaloid ryanodine, which is found mainly in the stem and roots of *Ryania speciosa*, as a potential insecticide, due to its paralyzing effects. Ryanodine leads to paralysis of cardiac and skeletal muscle by locking the RyR channel in a subconductance state, resulting in a constant SR Ca^{2+} leak. The high affinity binding of ryanodine to RyR was used to purify the receptor (Meissner, Henderson 1987).

RyRs are the largest known ion channels to date with a size of 2260 kDa, consisting of four 565 kDa monomers, each containing a transmembrane segment of about 10% of the protein sequence, whereas the other 90% form a giant cytoplasmic domain. Three mammalian isoforms of RyRs are known, encoded by three different genes. RyR1 is the isoform of skeletal muscle, while RyR2 is the main cardiac muscle isoform. RyR3 appears in brain and smooth muscle, but less so in striated muscle (Lanner et al. 2010).

RyR2 is closed at low cytosolic Ca^{2+} concentrations with open probability increasing at increasing [Ca^{2+}], binding to high affinity binding sites of the RyR2. Channel opening results in a cytosolic Ca^{2+} increase from 100 nmol/l to about 1 µmol/l, leading to activation of contractile proteins (Hasenfuss, Pieske 2002).

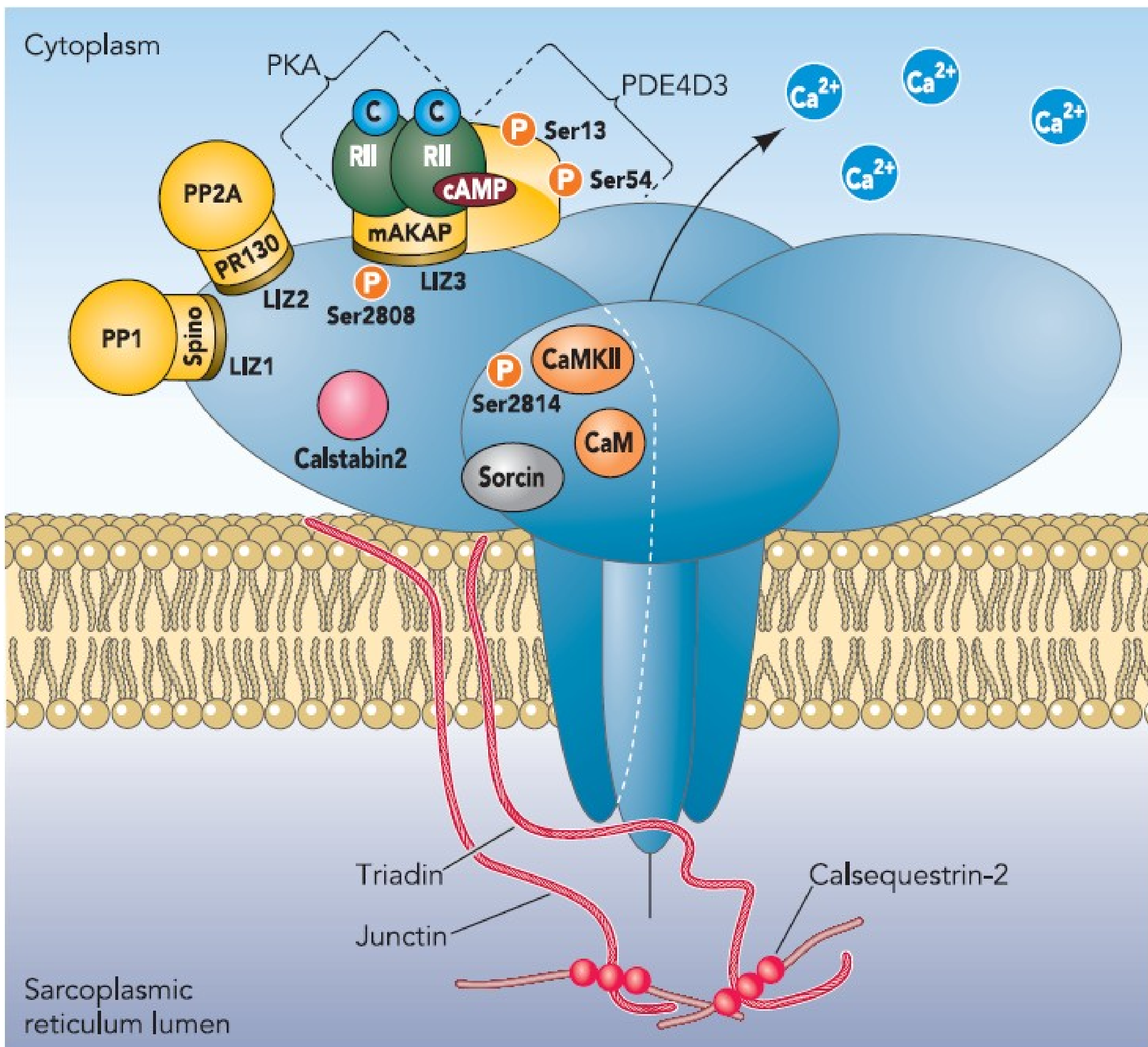


Figure 4: RyR2 macromolecular complex
The RyR2 macromolecular complex consists of four monomers, forming a tetrameric Ca^{2+} release channel. FKBP12.6 (calstabin2) and CaM are bound directly to the cytosolic side of the RyR2 monomers, while the binding of other regulatory proteins and enzymes is mediated by anchoring proteins. On the luminal side, triadin and junctin enable CASQ2 binding (Mohler, Wehrens 2007).

The cytoplasmic domain serves as a scaffold for regulatory subunits and enzymes. These modulatory proteins include calstabin2 (FKBP12.6), calmodulin (CaM), which help closing the channel after SR Ca^{2+} release and phosphodiesterase 4D3 (PDE4D3), which degrades cAMP, the activator of PKA.

During β-adrenergic stimulation RyR2 is phosphorylated by either PKA at residue serine 2808 (Ser2808) or by CaMKII at residue serine 2814 (Ser2814), leading to a channel activation due to an enhanced sensitivity to cytosolic Ca^{2+}. It has further been proposed, that PKA phosphorylation dissociates FKBP12.6 from RyR2, thus additionally increasing open probability (Marx et al. 2000, Kushnir, Marks 2010).

1.6.1.1 RyR2 in failing hearts

In the past years, pathological diastolic SR Ca^{2+} leak via dysfunctional RyR2 has been shown to play an important role in altered Ca^{2+} handling in HF. Discovery of over 150 RyR2 mutations causing arrhythmias and sudden cardiac death have added a new role for RyR2 dysfunction in cardiac disease (Priori, Chen 2011). The causes of the malfunction remain controversial and may involve a number of potential mechanisms:

- **FKBP12.6 dissociation**:

  FKBP12.6 (calstabin2) is a RyR2-binding protein that stabilizes the channel to prevent spontaneous SR Ca^{2+} release during diastole. Marx et al. were the first group to propose PKA phosphorylation of RyR2 to dissociate FKBP12.6 from the channel, thereby increasing RyR2 open probability (Marx et al. 2000). RyR2s from failing hearts were further shown to be PKA hyperphosphorylated (3-4 of the four RyR2 monomers are phosphorylated by PKA as compared to <1 in non-failing hearts) leading to a 60% reduction of FKBP12.6 associated with RyR2. While PKA protein levels are unchanged, PP1 and PP2A levels decrease, causing the hyperphosphorylation (Blayney, Lai 2009).

  This hypothesis was supported by a knock-in mouse model carrying a S2808A mutation site that could not be PKA phosphorylated. In WT mice, PKA treatment decreased FKBP12.6 binding to RyR2 and increased RyR2 open probability, which did not occur in S2808A mice (Wehrens et al. 2006). The hypothesis has been challenged by other groups, showing unchanged RyR2-FKBP12.6 interaction and no FKBP12.6 dissociation from the RyR2 due to PKA phosphorylation (Jiang et al. 2002).

- **Increased Ca^{2+} sensitivity**

  Despite reduced SR Ca^{2+} content, an increase in Ca^{2+} spark frequency has been demonstrated in failing hearts. In a chronic HF model, Kubalova et al. showed dramatically increased Ca^{2+} sensitivity of single RyR2s, causing increased rate of Ca^{2+} sparks and increased SR Ca^{2+} leak (Kubalova et al. 2005). This finding provided an explanation for the widely discussed

paradox of increased risk for DADs despite lower SR Ca^{2+} content. In line with this notion was a reduced 'store overload-induced Ca^{2+} release' threshold, caused by RyR2 mutations. HEK293 cells, expressing RyR2 mutations, located in the C-terminal, N-terminal or the central region of the channel, displayed an enhanced propensity for spontaneous Ca^{2+} release. The mutant RyR2 channels exhibited increased sensitivity to luminal but not to cytosolic Ca^{2+} without any differences in FKBP12.6 binding (Jiang et al. 2005).

- **Interdomain unzipping**

  Unzipping between the N-terminal and the central regions of RyR2 were proposed to hyperactivate the receptor, leading to SR Ca^{2+} leakage (Ikemoto, Yamamoto 2002). DPc10, a synthetic cardiac domain peptide, introduced by Oda et al., induced domain unzipping by destabilizing the interactions between these regions, but did not induce FKBP12.6 dissociation. However, the peptide facilitated the dissociation in response to PKA hyperphosphorylation, leading to prolonged Ca^{2+} transients and with smaller Ca^{2+} peak amplitudes. JTV19 was shown to inhibit the effects of domain unzipping and therefore Ca^{2+} leak, providing a possible new therapeutic agent against HF and arrhythmias (Oda et al. 2005).

1.6.1.2 RyR2 phosphorylation

RyR2 has two main phosphorylation sites, Ser2808 and Ser2814. Ser2808 is mainly phosphorylated by PKA although CaMKII is also able to phosphorylate it (Witcher et al. 1991, Rodriguez, Bhogal & Colyer 2003). On the other hand, Ser2814 is thought to be phosphorylated exclusively by CaMKII.

- **PKA phosphorylation**

Adrenergic stimulation, due to the so called 'fight or flight' response, increases intracellular cAMP, leading to PKA activation. Activated PKA can regulate all major ECC components, causing increased heart rate and contractile force (Blayney, Lai 2009). Different mechanisms for the PKA-dependent upregulation of SR Ca^{2+} release have been proposed, such as enhancement of I_{Ca} due to L-type

Ca^{2+} channel phosphorylation, activation of SERCA2a due to PLB phosphorylation or increased RyR2 sensitivity. The effects of phosphorylation on RyR2 itself have been proposed to consist of either synchronization of Ca^{2+} sparks, elevation of Ca^{2+} spark amplitude or to have little effect (Blayney, Lai 2009).

- **CaMKII phosphorylation**

$CaMKII_{\delta}$, the main cardiac isoform, is activated at high cellular [Ca^{2+}] and phosphorylates many of the same proteins involved in ECC as phosphorylated by PKA (Blayney, Lai 2009). CaMKII is activated by Ca^{2+} and CaM, which causes autophosphorylation and therefore Ca^{2+}-independent kinase activity (Ai et al. 2005). CaMKII-mediated phosphorylation of RyR2 leads to a higher open probability of RyR2 by sensitizing it to cytosolic Ca^{2+} (Wehrens et al. 2004).

Table 2: RyR2 expression and phosphorylation in HF

Protein	Group	HF model	Result
R2809	(Yeh et al. 2008)	dogs	=
R2809	(Fernandez-Velasco et al. 2009)	R4496C mouse model	=
R2809	(Belevych et al. 2007)	tachypaced dogs	=
R2809	(Jiang et al. 2002)	tachypaced dogs	=
R2814	(Fernandez-Velasco et al. 2009)	R4496C mouse model	=
R2814	(Netticadan et al. 2000)	rats post MI	↓
R2814	(Belevych et al. 2007)	tachypaced dogs	↑
RyR2	(Yeh et al. 2008)	Dogs	↓
RyR2	(Meyer et al. 1995)	human heart	=
RyR2	(Sainte Beuve et al. 1997)	human heart	=
RyR2	(Schillinger et al. 1996)	human heart	=
RyR2	(Fernandez-Velasco et al. 2009)	R4496C mouse model	=
RyR2	(Netticadan et al. 2000)	rats post MI	↓
RyR2	(Hu et al. 2011)	rats post MI	=
RyR2	(Jiang et al. 2002)	tachypaced dogs	=
RyR2	(Kubalova et al. 2005)	tachypaced dogs	↓

1.6.2 SR Calcium-ATPase

The SR Calcium-ATPase (SERCA2a) is a 110 kDa transmembrane protein, a family member of P-type ion translocating ATPases, which are essential in building ion gradients. SERCA pumps Ca^{2+} ions against a large concentration gradient, from the cytoplasm into the SR (Erkasap 2007). So far, three different SERCA genes (SERCA1-3) have been identified, with SERCA2a being the dominant isoform in cardiac muscle, where the pump is not only determining the electrical, but also the contractile properties (Pavlovic et al. 2005). Two Ca^{2+} ions are transported into the SR by the pump for each ATP molecule consumed. Since the amount of Ca^{2+} released by the SR depends on $[Ca^{2+}]_{SR}$ as well as the Ca^{2+} gradient, SERCA2a is not only determining the rate of relaxation but also the amount of Ca^{2+} available for the next contraction, therefore regulating rate and amplitude of contraction (Leszek et al. 2007, Vandecaetsbeek et al. 2009).

1.6.2.1 SERCA2a in failing hearts

SR Ca^{2+} uptake has consistently been shown to be decreased in different models of HF, resulting in slowed relaxation and $[Ca^{2+}]_i$ decline typically observed in HF (Bers 2001). SERCA2a mRNA was shown to be reduced in hypertrophied and failing human heart, while SERCA2a protein expression was significantly decreased only in failing hearts, suggesting a causal relation between SERCA2a protein reduction and the development of myocardial failure (DiPaola et al. 2001). Moreover, increasing SERCA2a expression by adenoviral gene transfer has been shown to accelerate relaxation and $[Ca^{2+}]_i$ decline (Sakata et al. 2007).

Table 2: SERCA2a expression in HF

Group	HF model	Result
(Kiss et al. 1995)	guinea pigs post TAC	↓
(DiPaola et al. 2001)	human heart	↓
(Meyer et al. 1995)	human heart	↓
(Movsesian et al. 1994)	human heart	=
(Schillinger et al. 1996)	human heart	↓
(Schmidt et al. 1999)	human heart	=
(Netticadan et al. 2000)	rats post MI	↓
(Jiang et al. 2002)	tachypaced dogs	↓
(Kubalova et al. 2005)	tachypaced dogs	=

1.6.3 Phospholamban

Phospholamban (PLB) is a key regulatory protein of SERCA2a. PLB is an acid transmembrane protein expressed predominantly in cardiac muscle, inhibiting only isoforms SERCA1 and SERCA2, but not SERCA3 (Bers 2001). Because SERCA2a activity is crucial for maintaining cardiac Ca^{2+} homeostasis, its regulation through PLB has a significant impact on calcium cycling.

PLB forms a pentamer, consisting of 5 identical subunits, each containing 52 amino acids, which are organized into three domains, including domain Ia, Ib and II. Domain Ia (amino acids 1-20) contains two distinct phosphorylation sites at residues serine 16 (Ser16) and threonine 17 (Thr17); domain Ib includes amino acids 21-30 and domain II (amino acids 31-52) traverses the SR membrane (Simmerman, Jones 1998, MacLennan, Kranias 2003).

When unphosphorylated, PLB inhibits SERCA2a by lowering its apparent Ca^{2+} affinity, without alteration of the maximal pumping rate. Phosphorylation of PLB plays an important part in cardiac Ca^{2+} cycling. The two main phosphorylation sites, Ser16 and Thr17, can be phosphorylated by PKA and CaMKII, respectively. An increase in phosphorylation results in relief of inhibition, enhancing SR Ca^{2+} uptake, which is predominantly responsible for the effects of β-adrenergic stimulation on cardiac relaxation (Minamisawa, Sato & Cho 2004).

1.6.3.1 PLB in failing hearts

In HF, PLB protein expression levels do not differ from non-failing myocardium (Simmerman, Jones 1998). However, a decrease in SERCA2a protein in HF results in an increased PLB:SERCA ratio, leading to an unproportionally high SERCA2a inhibition, which results in a lower SERCA2a Ca^{2+} affinity and decreased SR Ca^{2+} content. This further decreases Ca^{2+} release from the SR during systole, causing impaired Ca^{2+} homeostasis and contractile dysfunction, ultimately leading to the HF phenotype.

PLB phosphorylation has been shown to be decreased in HF, resulting in an even greater SERCA2a inhibition, which may in part be due to the increased global phosphatase expression seen during HF (Netticadan et al. 2000, Sande et al. 2002, Minamisawa, Sato & Cho 2004).

Due to its ability to modulate SERCA2a-mediated Ca^{2+} uptake, SR Ca^{2+} load and myocyte Ca^{2+} cycling, PLB is a critical regulator of basal SR Ca^{2+} handling and contractility in the heart, and might therefore be a promising target to improve systolic and diastolic function in failing myocardium (Gregory, Kranias 2006).

Table 3: PLB expression and phosphorylation in HF

Protein	Group	HF model	Result
PLB	(Kiss et al. 1995)	guinea pigs post TAC	↓
PLB	(Dash et al. 2001)	human heart	=
PLB	(Jiang et al. 2002)	human heart	=
PLB	(Movsesian et al. 1994)	human heart	=
PLB	(Schmidt et al. 1999)	human heart	=
PLB	(Hu et al. 2011)	rats post MI	=
PLB	(Netticadan et al. 2000)	rats post MI	↓
PLB	(Jiang et al. 2002)	tachypaced dogs	↓
PLB	(Kubalova et al. 2005)	tachypaced dogs	=
Ser16	(Dash et al. 2001)	human heart	↓
Ser16	(Schmidt et al. 1999)	human heart	↓
Ser16	(Hu et al. 2011)	rats post MI	↓
Ser16	(Netticadan et al. 2000)	rats post MI	=
Ser16	(Sande et al. 2002)	rats post MI	↓
Thr17	(Yeh et al. 2008)	dogs	↑
Thr17	(Dash et al. 2001)	human heart	↓
Thr17	(Hu et al. 2011)	rats post MI	↓
Thr17	(Netticadan et al. 2000)	rats post MI	↓
Thr17	(Sande et al. 2002)	rats post MI	=

1.6.4 Calsequestrin

Calsequestrin (CASQ) is the major Ca^{2+}-binding protein within the SR. The cardiac isoform CASQ2 is a 55 kDa protein with low Ca^{2+} affinity and high capacity (each molecule can bind 35-40 Ca^{2+} ions or 800-900 nmol of Ca^{2+}/ mg CASQ). CASQ is very acidic and is present as a mixture of monomers, dimers and multimers within the SR. CASQ2 polymerization is Ca^{2+}-dependent, such that at high Ca^{2+} concentrations monomers self assemble into dimers and polymers, whereas at low concentrations polymers break into monomers. The polymerization-depolymerization process has been shown to be too slow to occur dynamically on a beat-to-beat basis (within minutes rather than seconds) (Terentyev et al. 2008). Whereas the CASQ2 multimers represent the high Ca^{2+} binding form and serve as a storage reservoir, Györke et al. reported CASQ2 monomers to interact with the RyR2 complex via triadin and junctin to serve as a luminal Ca^{2+} sensor. CASQ2 is the actual Ca^{2+} sensor for RyR2, while triadin and junctin are required to physically link CASQ2 to RyR2, inhibiting its activity at low luminal Ca^{2+} concentrations. This inhibition is gradually relieved on rising luminal [Ca^{2+}], as the Ca^{2+}-binding sites on CASQ2 become more and more occupied. This weakens the interactions of CASQ2 with triadin and junctin, therefore increasing RyR2 channel open probability. The channel is maximally active when CASQ2 dissociates completely from the RyR2 complex at Ca^{2+} concentration of 5 mM (Gyorke et al. 2004, Terentyev et al. 2008, Gyorke 2009).

1.6.4.1 Calsequestrin in failing hearts

CASQ2 expression has been reported to be unchanged in failing human myocardium (Fan et al. 2004, Movsesian et al. 1994) and failing murine hearts (Movsesian et al. 1994), indicating that at lower $[Ca^{2+}]_{SR}$ in HF, free $[Ca^{2+}]_{SR}$ is reduced as well, suggesting less Ca^{2+} being available for release (Bers 2001).

In genetically altered mice with a complete CASQ2 deletion, animals presented with normal cardiac function under baseline conditions (Knollmann et al. 2006).

Table 4: CASQ2 expression in HF

Group	HF Model	Result
(Yeh et al. 2008)	dogs	↓
(Dash et al. 2001)	human heart	=
(Meyer et al. 1995)	human heart	=
(Movsesian et al. 1994)	human heart	=
(Schillinger et al. 1996)	human heart	=
(Hu et al. 2011)	rats post MI	↓
(Kubalova et al. 2005)	tachypaced dogs	=

1.7 Catecholaminergic polymorphic ventricular tachycardia

CPVT is a ‘highly malignant form of arrhythmogenic disorder characterized by exercise or emotional stress-induced ventricular tachycardia in the absence of structural heart disease’ (Liu, Ruan & Priori 2008). CPVT was initially described by Leenhardt et al., who reported a seven year follow up of 21 children with CPVT and described it as ‘idiopathic, potentially lethal ventricular tachyarrhythmia in children’ (Leenhardt et al. 1995). In 2001 four single nucleotide substitutions leading to missense mutations in the RyR2 were discovered in four patients with clinical CPVT, causing the more frequent autosomal dominant form of CPVT (Priori et al. 2001). Shortly thereafter, a CASQ2 missense mutation was identified in a large consanguineous Bedouin family affected by the recessive form of CPVT, which accounts for about 1-2% of CPVT patients (Lahat et al. 2001).
In only about 50-70% of CPVT patients mutations in RyR2 or CASQ2 have been found, suggesting the existence of further, unknown CPVT-associated genes (Liu, Ruan & Priori 2008).

1.7.1 Clinical presentation

Affected individuals usually develop syncope, triggered by emotional or physical stress, as a first clinical symptom. Approximately 30% of CPVT patients show symptoms before the age of 10; whereas 79% experience at least one syncopal episode before the age of 40 (Mohamed, Napolitano & Priori 2007, Cerrone, Napolitano & Priori 2009). Because CPVT patients show no abnormalities in resting ECG, cardiac structure and function, average delay of diagnosis is about two years. The most important test is a graded exercise test, by which ventricular tachyarrhythmias are induced (Figure 5). If patients are more perceptive to emotional stressors, Holter monitoring may be useful (Cerrone, Napolitano & Priori 2009). A family history of juvenile sudden cardiac death (SCD) was reported in over 30% of 30 CPVT patients examined, therefore a detailed family anamnesis was suggested to help establish the right diagnosis (Priori et al. 2002).

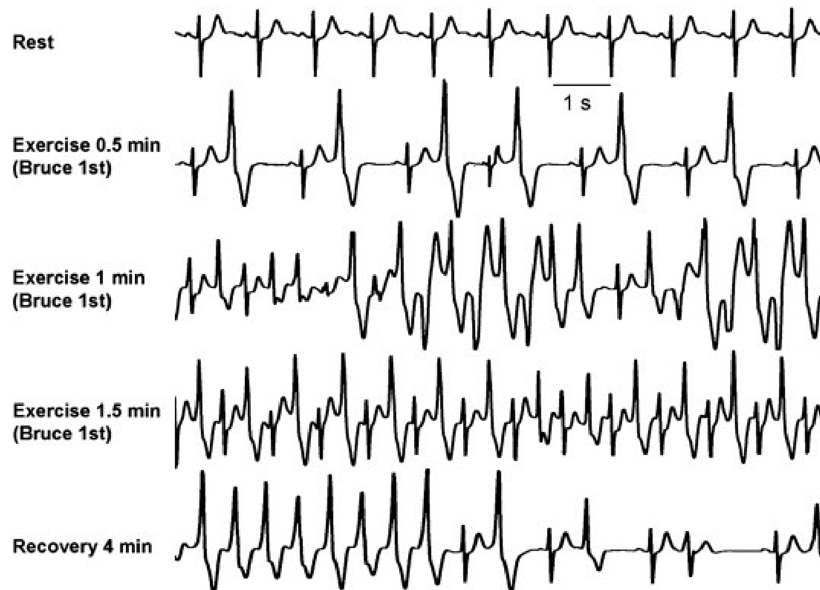


Figure 5: Exercise stress test in a patient with CPVT
Exercise stress test in a patient with polymorphic VT and a RyR2 mutation. VTs can be observed with progressive worsening during exercise. Typical bidirectional VT develops after 1 minute of exercise with a sinus heart rate of approximately 120 bpm. After the exercise, arrhythmias quickly recede. Adopted from (Liu, Ruan & Priori 2008)

Despite presenting with a normal resting ECG, CPVT patients typically show progressive arrhythmias with increasing exercise intensity. Arrhythmias are characterized by a rotation of the QRS axis of 180°, alternating on a beat-to-beat basis and an increase in complexity can be observed with higher exercise intensity (Liu, Ruan & Priori 2008).

1.7.2 Molecular mechanisms of CPVT

1.7.2.1 RyR2 mutations and CPVT

At the time of writing, over 150 different RyR2 mutations have been identified and linked to CPVT (Priori, Chen 2011), with most of them being missense mutations. Several different molecular mechanisms have been proposed to explain how the mutations lead to uncontrolled Ca^{2+} release, which further enhances the induction of DADs.

The first hypothesis includes defective luminal Ca^{2+} activation leading to a reduced RyR2 SR Ca^{2+} release threshold, a process termed enhanced “store overload-induced Ca^{2+} release” (SOICR). In CPVT-associated RyR2 mutants expressed in HEK-293 cells, a reduction in SOICR threshold was shown. Furthermore, RyR2

mutations primarily increased the channel sensitivity to luminal, but not cytosolic Ca^{2+} (Jiang et al. 2005). In line with this notion, a higher incidence of Ca^{2+} sparks in resting and paced $RyR2^{R4496C+/-}$ cardiac myocytes was reported, indicating an enhanced Ca^{2+} sensitivity (Fernandez-Velasco et al. 2009).
The second hypothesis proposed FKBP12.6 dissociation due to increased PKA phosphorylation, causing enhanced RyR2 open probability (Marx et al. 2000). A third hypothesis explains the increased SR Ca^{2+} leak by defective interdomain interactions, leading to hyperactivation of RyR2 (Ikemoto, Yamamoto 2002).

1.7.2.2 CASQ2 mutations and CPVT

The recessive form of CPVT is caused by a missense mutation in the cardiac calsequestrin gene. So far, seven different mutations have been discovered (Gyorke, Terentyev 2008). CASQ2 is not only important for binding Ca^{2+} within the SR, it is also a crucial modulator of RyR2 open probability. Therefore, mutations causing reduced or defective expression of CASQ2 not only decrease the number of luminal Ca^{2+} binding sites, increasing free intra SR [Ca^{2+}], but also reduce the ability of CASQ2 to inhibit RyR2 activity (Gyorke 2009). Both mechanisms disrupt the control of RyR2 to prevent spontaneous Ca^{2+} release and they are likely to act in concert to induce the CPVT phenotype.

1.7.2.3 Mouse models

The first CPVT transgenic knock-in mouse model reproducing the human RyR2-R4497C mutation was created by the Priori group (Cerrone et al. 2005). The mouse model carries a $RyR2^{R4496C}$ mutation, presenting a phenotype with the typical bidirectional VT. DADs develop in paced $RyR2^{R4496C}$ myocytes even without β-adrenergic stimulation, explaining why beta-blocker treatment offers only incomplete protection for CPVT patients. Thereafter, other mouse models with a similar phenotype were generated, such as mice harboring $RyR2^{R176Q}$ or $RyR2^{R2474S}$ mutations (Kannankeril et al. 2006, Lehnart et al. 2008).

1.7.3 Therapeutic options

Beta-blockers have been the main pharmacological therapy for CPVT patients, with nadolol and propranolol being the most widely used. The highest dose tolerated is given to maximize arrhythmia control, but still about 30% of CPVT patients experience arrhythmias or syncope during therapy, indicating the necessity of an implantable cardiac defibrillator (ICD) (Cerrone, Napolitano & Priori 2009). While beta-blockers are indicated as the primary prevention, ICDs should be considered for secondary prevention in all patients with recurrence of syncope or VT or after cardiac arrest. Fifty percent of CPVT patients with an ICD received an appropriate shock during a 2 year follow up (Liu, Ruan & Priori 2008). At present, therapy remains inadequate.

1.8 Hypothesis

In the present study, we investigated whether increased SR Ca^{2+} leak caused by the $RyR2^{R4496C+/-}$ mutation changes the expression of Ca^{2+} handling proteins during the development of pressure overload-induced hypertrophy and the progression to HF.

We employed immunoblotting to quantify (1) the expression of SERCA2a, PLB and CASQ2 and (2) phosphorylation levels of RyR2 and PLB at PKA-dependent phosphorylation sites RyR2-Ser2808 and PLB-Ser16, as well as CaMKII-dependent sites RyR2-Ser2814 and PLB-Thr17, respectively. Potential changes in the expression and/or phosphorylation levels of the Ca^{2+} regulatory proteins studied may imply the activation of SR Ca^{2+} leak-dependent signaling pathways involved in the progression of hypertrophy and HF.

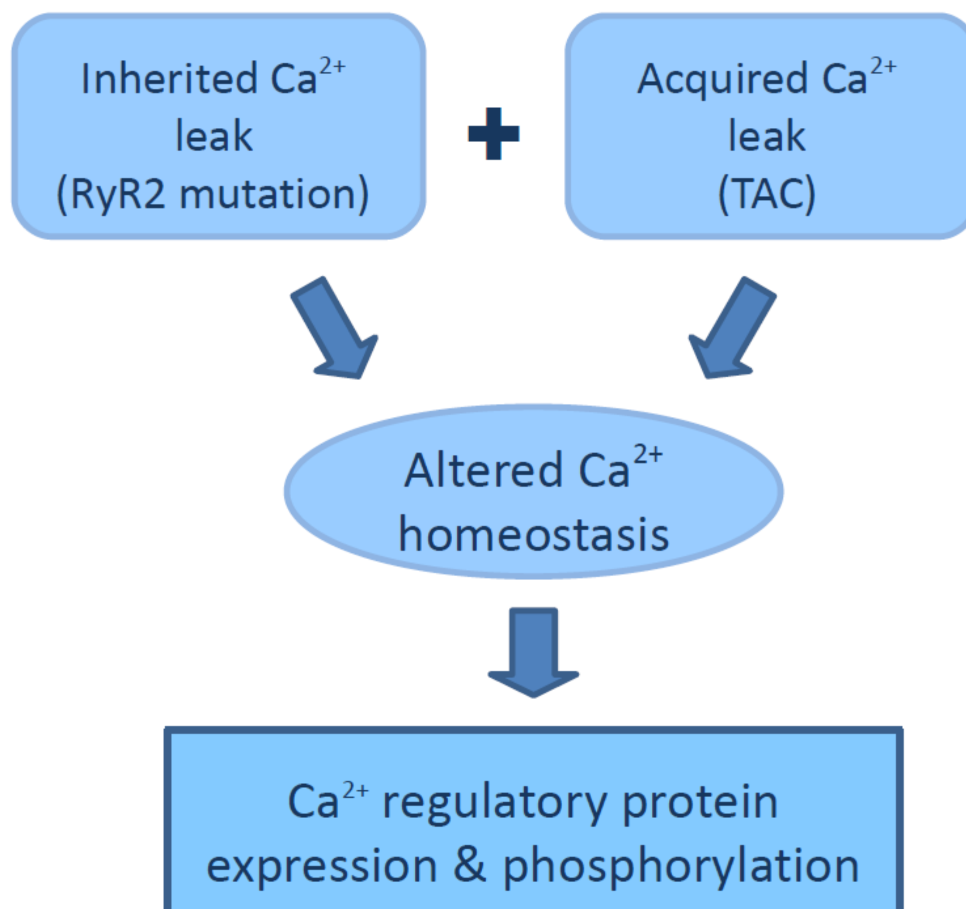


Figure 6: Schematic illustration of our hypothesis

An increased SR Ca^{2+} leak via a dysfunctional RyR2, caused by a mutation and chronic pressure overload, underlies altered Ca^{2+} homeostasis, which affects the expression and phosphorylation of Ca^{2+} handling proteins.

2. Materials and Methods

2.1 Mice

Hearts from RyR2$^{R4496C+/-}$ knock-in mice (R4496C) harboring a human CPVT-associated RyR2 mutation and their WT littermates (C57/B6 strain; male and female) were used to determine the expression and phosphorylation level of Ca^{2+} handling proteins, including RyR2, SERCA2a, PLB and CASQ2. All mice underwent a surgical procedure as described below.

2.2 Minimally invasive transverse aortic constriction (TAC)

Aortic banding is a method used to induce left ventricular hypertrophy (LVH) in mice, mimicking human aortic stenosis with development of pressure overload-induced LVH. We performed minimally invasive aortic constriction as described elsewhere (Hu et al. 2003), placing the ligature on the transverse portion of the aorta. The surgery was performed by the investigator blinded to the genotype (Dr. Simon Sedej). In our study, WT and R4496C mice (male: 24±1 g; female: 21±1 g) were anesthetized using subcutaneous injections of ketamine and xylazine (100 mg/kg and 5 mg/kg body weight, respectively). After a horizontal incision at the jugulum, the transverse aorta was displayed and a 27-gauge blunted needle was tied parallel to the aorta using a 5-0 non-absorbable suture. Then, the needle was removed, the skin was closed and the mice were kept on a warming pad at 37°C throughout the experiment, until full recovery from anesthesia. Sham animals underwent the same surgical procedure except for the aortic constriction. One week post surgery, mice were sacrificed, euthanized by anesthesia using isoflurane-rich atmosphere followed by a cervical dislocation. Hearts were rapidly excised and washed in ice-cold PBS solution. After removal of both atria and the right ventricle, left ventricles were flash frozen in liquid nitrogen.

The consistency of the TAC procedure was validated by echocardiography performed one and three weeks post surgery (Dr. Albrecht Schmidt). Preliminary data showed WT-TAC mice to exhibit concentric LVH with preserved cardiac performance one week after TAC, whereas R4496C-TAC mice developed

eccentric hypertrophy and overt HF phenotype, characterized by chamber dilatation and reduced ejection fraction.

2.3 Homogenization

Small pieces of flash-frozen left ventricles from murine hearts were homogenized on ice with freshly prepared homogenization buffer (HB) (Table 5).

Table 5: Homogenization buffer

Reagent	Final concentration
NaCl	137 mM
Tris HCl (pH=7)	20 mM
NP40	1%
Glycerol	10%
NaF	20 mM
Sodiumpyrophosphate	1 mM
β-Glycerophosphate	50 mM
EDTA (pH=8)	10 mM
EGTA (pH=7)	1 mM
PMSF	1mM
Na_3VO_4	1mM
Aprotinin	4 µg/ml
Leupeptin	4 µg/ml
Pepstatin A	4 µg/ml
dd H_2O	

Homogenization was performed manually using micro tissue grinders. The samples were then centrifuged for 3 minutes at 5000 rounds per minute (RPM). The supernatant was collected and centrifuged together with the re-homogenized pellet. After another 3 minutes of centrifugation, this time at 4°C and 13000 RPM,

the supernatant was again collected. Further dilution with additional HB was performed to keep the final protein concentration of the samples below the concentration of the highest BCA standard. This ensured that samples were within the range of the regression line, of which values were calculated from the standards.

2.4 BCA protein assay

The protein concentration of each sample was determined using bicinchoninic acid (BCA) protein assay (Thermo Fisher Scientific Inc.). The BCA assay is based on the reduction of Cu^{2+} to Cu^{1+} in a temperature-dependent reaction of $CuSO_4$ with the protein's peptide bonds known as *biuret reaction*, characterized by a light blue complex. The amount of reduced Cu^{1+} is directly proportional to the amount of protein in the solution. Each reduced Cu^{1+} ion then chelates with two molecules of bicinchoninic acid, forming an intense purple colored reaction product, which can be quantified using spectrometric analysis (Thermo Fisher Scientific Inc.).

2.4.1 BCA assay protocol

Six different BSA (bovine serum albumin) standards from the 3 g/l BSA stock solution, consisting of 3 mg BSA dissolved in 1 ml HB (Table 6), were prepared.

Table 6: BSA standards

Final concentration (µg/µl)	Homogenization buffer (µl)	BSA stock solution (µl)
0	100.00	0.00
0.5	83.33	16.67
1.0	66.67	33.33
1.5	50.00	50.00
2.0	33.33	66.67
2.5	16.67	83.33
3.0	0.00	100.00

The BCA standard working reagent (SWR) was prepared according to the manufacturer's instructions: 50 volume parts of BCA™ Reagent A (consisting of Na_2CO_3, $NaHCO_3$, BCA and sodium tartrate in 0.1 M NaOH) were mixed with 1 volume part of BCA™ Reagent B (containing 4% $CuSO_4$), resulting in a light green solution stable at RT for 1 week.
One volume part of each BSA standard was added to 50 volume parts of SWR, while for the unknown homogenates one volume part of the sample was prepared by diluting the homogenates with HB using the ratios (volume parts) 1:9, 2:8 and 5:5, before 50 volume parts of SWR were added.
All samples were then incubated in a water bath at 37°C for 30 minutes (for a working range of total protein concentration of 20-2000 µg/ml). After cooling the samples to RT, the spectrometer was set to 562 nm and zeroed with the blank standard (HB only) before the absorbance of the standards as well as of the samples was measured. Based on a regression line calculated from the BSA standards, total protein concentrations of the samples were determined. Finally, an average of all three samples for each homogenate was calculated.

2.5 Western Blot

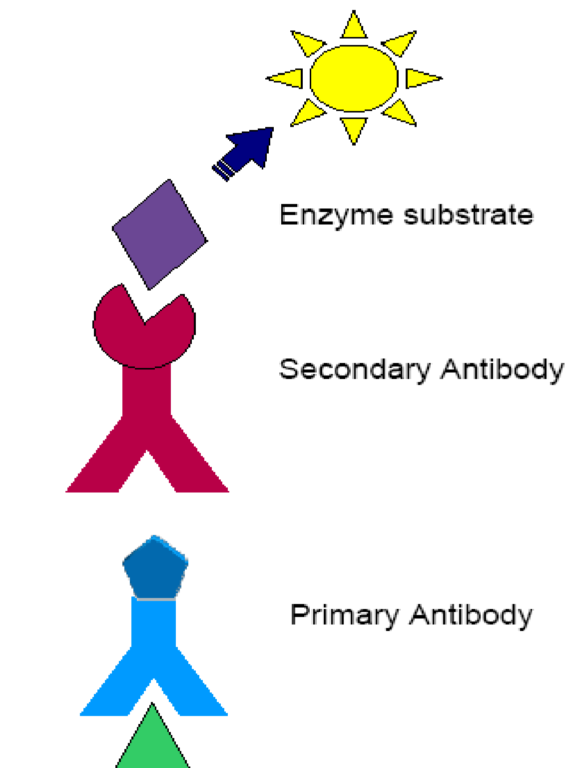


Figure 7: Antibody binding

2.5.1 Background

Western blotting is a conventional biochemical technique to identify and quantify different proteins in cell extracts using antigen-antibody binding (also called immunoblotting). Native or denatured proteins are first separated by gel electrophoresis using an electrical current to drive the proteins through a polymer matrix, separating them according to their molecular weight, 3-D structure and polypeptide length. A marker, producing bands of a known size, is used to help identify proteins of interest. Electric current is then used to transfer proteins onto a nitrocellulose membrane, which immobilizes the proteins and renders them accessible to antibodies. Once the proteins are bound to the membrane, a specific antibody, binding to the protein of interest, is added. A secondary antibody, binding the primary antibody, is linked to an enzyme, which, after adding the enzyme's substrate, allows for visualization of the protein by producing chemiluminescence, enabling photographing onto X-ray films (Thermo Fisher Scientific Inc.).

2.5.2 Reagents

- Homogenization buffer (see Table 2 for details)
- XT sample buffer 4x (BIO-RAD, USA)
- XT reducing buffer (BIO-RAD, USA)
- Ponceau S (Sigma-Aldrich, USA)
- 10x TBST buffer (147.43mM NaCl; 2.5M Tris-base, adjust pH to 7,5; add 1% tween)

- 5% non fat dry milk powder in 1x TBST
- SuperSignal West PICO Chemiluminescent Substrate (Thermo Scientific, USA)
    - SuperSignal® West Pico Stable Peroxide Solution (Thermo Scientific, USA)
    - SuperSignal® West Pico Luminol Enhancer Solution (Thermo Scientific, USA)

- Antibodies:

Table 7: Primary antibodies

Antibody	Species	Company	Cat. Nr.	PAD	SAD
Anti-GAPDH	Mouse	HyTest Ltd., FIN	5G4 MAB 6C5	1:55 000	1:10 000
Anti-CASQ	Rabbit	Thermo Scientific, USA	PA1-913	1:1000	1:10 000
Anti-SERCA2a	Rabbit	Badrilla, UK	A010-20	1:5000	1:10 000
Anti-RyR2 P-Ser2814	Rabbit	Badrilla, UK	A010-31	1:5000/ 1:1000	1:5000
Anti-RyR2 P-Ser2808	Rabbit	Badrilla, UK	A010-30	1:5000	1:5000
Anti-RyR2	Mouse	Pierce Antibodies, USA	MA3-925	1:5000	1:5000
Anti-PLB P-Ser16	Rabbit	Badrilla, UK	A010-12	1:1000	1:5000
Anti-PLB P-Thr17	Rabbit	Badrilla, UK	A010-13	1:3000	1:5000
Anti-PLB A1	Mouse	Badrilla, UK	A010-14	1:1000	1:5000

PAD: primary antibody dilution
SAD: secondary antibody dilution

Table 8: Secondary antibodies

Secondary Antibodies	Species	Company	Cat.Nr.
ECL Anti-Mouse IgG Horseradish Peroxidase-linked species-specific whole Antibody	Sheep	GE Healthcare, USA	NA931
ECL Anti-Rabbit IgG Horseradish Peroxidase-linked species-specific whole Antibody	Donkey	GE Healthcare, USA	NA934

2.5.3 Western Blot Equipment and Materials

- Whatman Protran BA 85 (BA 79 for PLB) Nitrocellulose Transfer Membrane (Whatman group, UK)
- Criterion XT 4-12% Bis-Tris Precast gel (BIO-RAD, USA)
- Criterion 16.5% Tris-Tricine Precast gel (for PLB blots) (BIO-RAD, USA)
- Electrophoresis tanks (BIO-RAD, USA)
- Transfer sandwich with filter paper and sponge

2.5.4 Western Blot protocol

Homogenates were diluted with HB, 4x sample buffer and 20x reducing agent. A 4-12% XT Bis-Tris gel (16.5% Tris-Tricine gel for PLB blots) was loaded with the samples and electrophoresis was performed at 70 V and 100 V for 20 and 130-160 minutes, respectively.

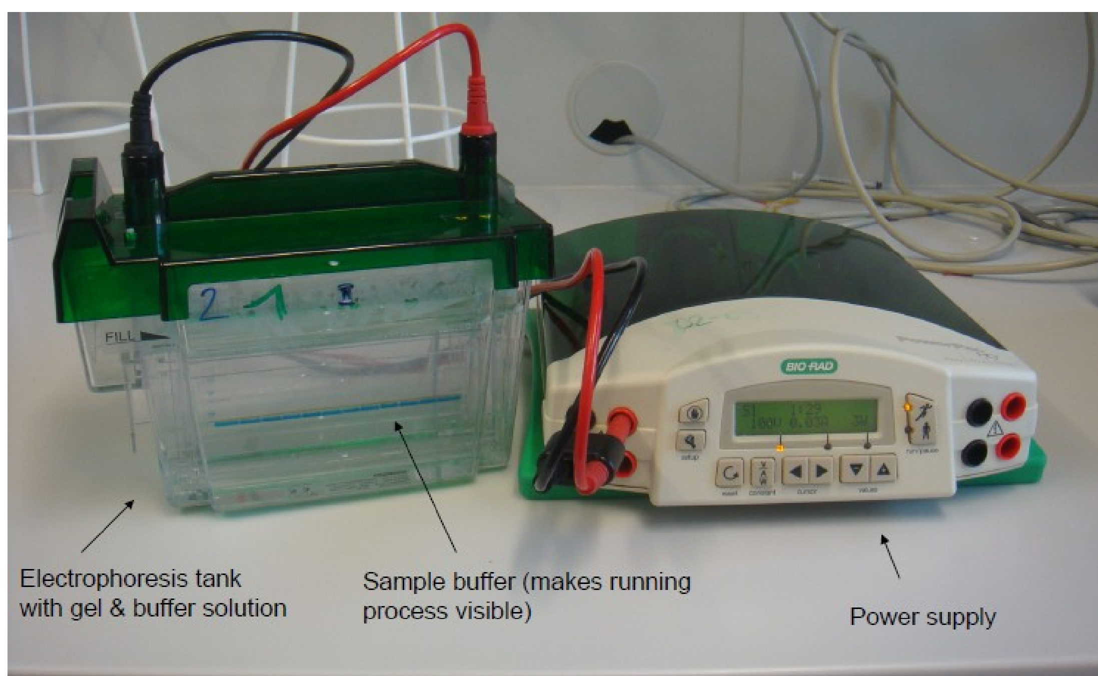


Figure 8: Gel electrophoresis

Next, proteins were transferred onto a nitrocellulose transfer membrane (for preparation of the transfer sandwich see Fig. 9) using gel electrophoresis of 150 mA at 4°C for two hours followed by 80 mA at 4°C overnight.

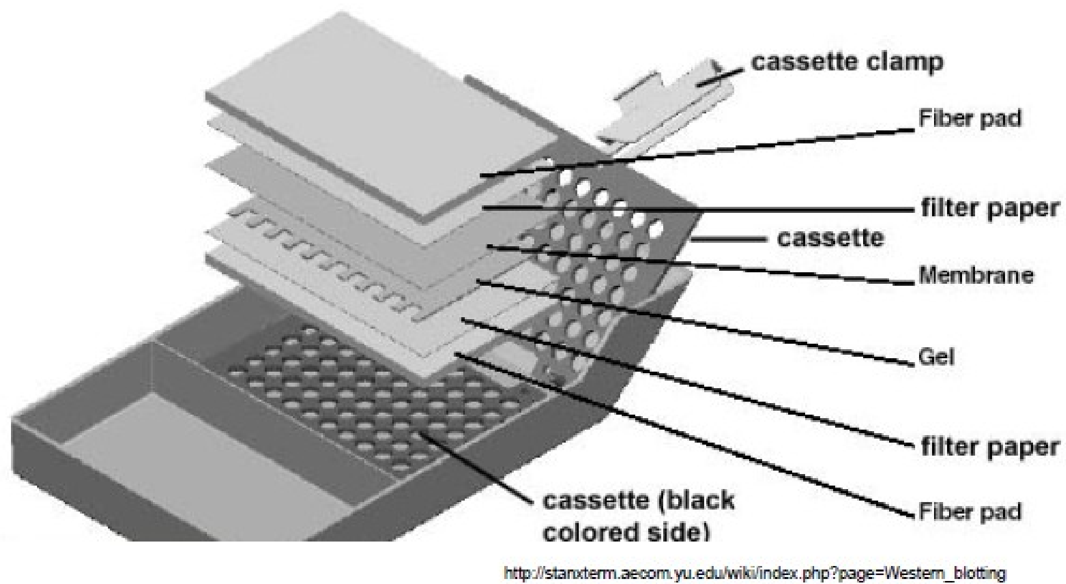


Figure 9: Preparing the transfer sandwich

After transfer, membranes were stained with Ponceau-S for visualization of the proteins. Ponceau-S is a negatively charged dye that binds reversibly to the positive amino groups of proteins, therefore enabling visualization of successful transfer.

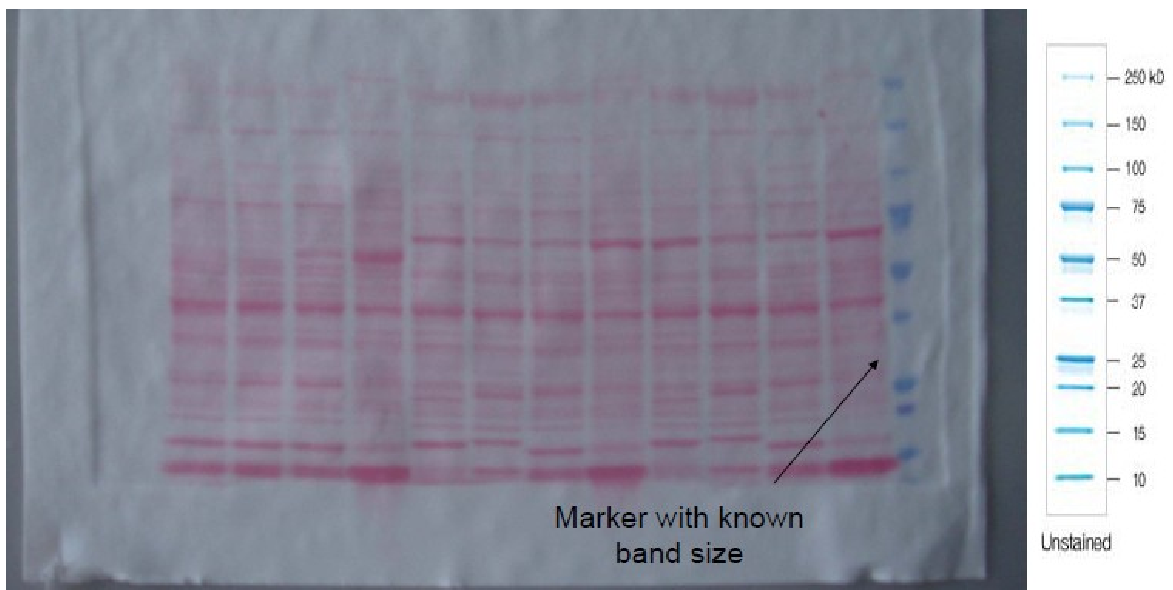


Figure 10: Membrane stained with Ponceau-S dye after protein transfer

Non-specific binding sites were blocked using 5% milk for one hour at RT. Membranes were then washed twice for 10 minutes with 1x TBST buffer and incubated with the primary antibody at 4°C overnight.

To remove unbound antibodies, membranes were washed three times with 1x TBST buffer for 10 minutes. Membranes were then incubated with the secondary antibody for 50 minutes at 4°C, which was afterwards removed by washing the membranes four times for 10 minutes using 1x TBST buffer.
PICO chemiluminescence working solution (CWS) was prepared according to the manufacturer's protocol by mixing equal volume amounts of stable peroxide and luminol/enhancer solution (light-sensitive). After incubation with CWS for 5 minutes, membranes were put into photo cassettes and exposed to X-ray films.

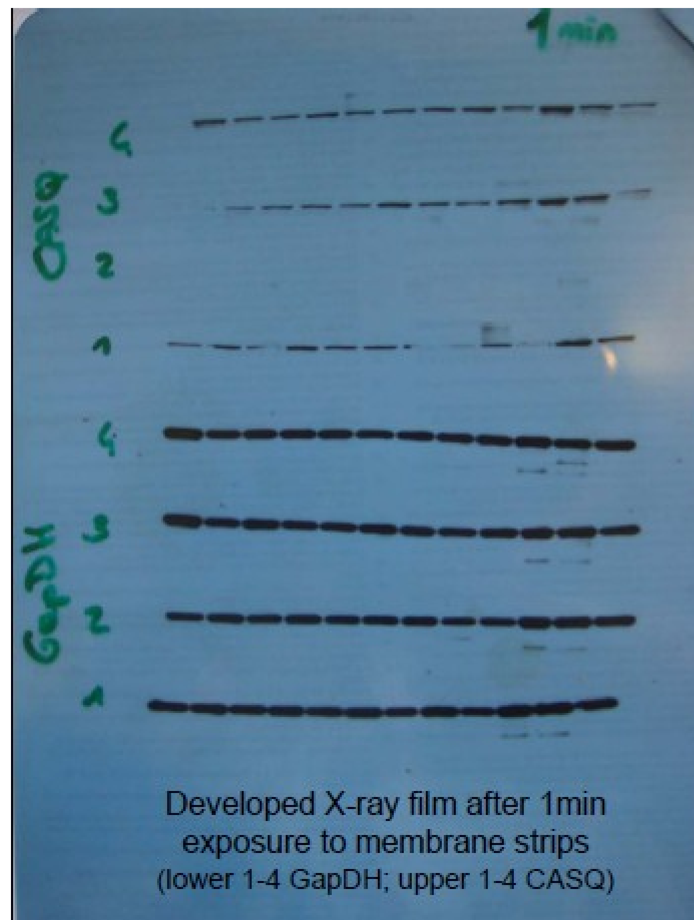


Figure 11: Developed X-ray film with protein bands

Exposure time varied depending on antibody concentration and prolonged with time, because CWS luminescence gradually diminishes. Afterwards membranes were washed with ultra pure water and dried for ten minutes at 60°C for archivation. When quantifying fractional phosphorylation of a certain protein, membranes were reprobed. This enabled us to work with the same amount of total protein and allowed for the normalization. For this purpose, antibodies were

stripped off by adding 0.2 M NaOH for 8 minutes between two washing steps (4 minutes each) using ultra pure water. Binding sites were then again blocked with 5% milk for an hour at RT and incubated with primary antibodies at 4°C overnight.

2.5.5 Detection of weak proteins (weak expression and/or weak phosphorylation)

If protein signals on the X-ray films were weak or non-detectable even after long exposure times, Quenix^{TM} Western Blot Signal Enhancer or Super Signal West Femto Maximum Sensitivity Substrate (both Thermo Scientific, USA) were used to improve detection.

<u>1. Quenix^{TM} Western Blot Signal Enhancer</u>

The signal enhancer promotes the antigen-antibody interaction, resulting in increased detection efficiency. Its broad application and the absence of enhancement of background signals are its major advantages. The enhancer's incompatibility with Ponceau-S, however, disables the visualization of proteins after the transfer.

Protocol: According to the manufacturer's instructions, membranes were rinsed using ultra pure water and incubated with Reagent 1 for two minutes. Following another rinsing step, Reagent 2 was applied for 10 minutes. After a final rinsing step, membranes were blocked with 5% milk and incubated using primary antibody at 4° C overnight (see also page 31).

<u>2. Super Signal West Femto Maximum Sensitivity Substrate</u>

The femto substrate is extremely sensitive to even smallest amounts of antigen. However, it enhances background signals and works only with HRP-linked detection systems.

Protocol: The solution was prepared by mixing equal amounts of Reagent A and B and diluted 1:2 with 1x TBST buffer. The membranes were then incubated for 5 minutes with this substrate instead of CWS.

When proteins are expressed at very low concentrations, combining both aforementioned methods (the enhancer and femto substrate) is advantageous.

2.5.6 Protein analysis

Films were scanned with Chemi Doc TM XRS Universal Hood (BIO-RAD, USA) and detection bands were quantified by using Quantity One™ software (BIO-RAD, USA). Densitometric values of the pool were used to generate a regression line, from which protein concentrations of the samples were calculated. Expression levels of CASQ2, SERCA2a and PLB were normalized to GAPDH (Glyceraldehyde 3-Phosphate Dehydrogenase). GAPDH was used as a loading control protein because of its stable expression in all tissue types. Normalization to GAPDH avoids false result interpretation due to differences in protein loading on the gel. Phosphorylation levels of RyR2 and PLB were normalized to the total protein detected in the same sample. This approach was used, because measurements of absolute phosphorylation levels may not accurately reflect the proportion of protein phosphorylated.

2.6 Statistical analysis

Data are shown as mean ± standard error of the mean (S.E.M.). Statistical differences between data sets were considered significant when $P<0.05$. Statistical analysis was performed by two-way analysis of variance (ANOVA) using SigmaPlot and SigmaStat (Systat Software Inc., USA). After detecting an overall significance, the Holm-Sidak post hoc test was performed.

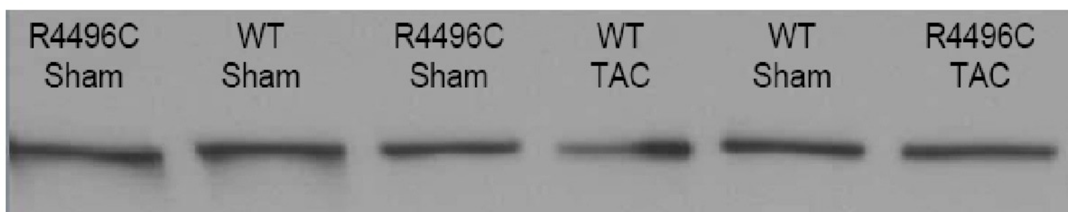
3. Results

3.1 Ryanodine receptor type 2

3.1.1 RyR2 phosphorylation at Ser2808

RyR2 phosphorylation level was determined using phosphorylation site-specific antibodies. Results were normalized to total RyR2 protein expression for each sample. Fractional RyR2 phosphorylation at residue Ser2808 showed no significant alterations between the groups (WT-sham: 1.000 ± 0.07; WT-TAC: 0.889 ± 0.06; R4496C-sham: 1.031 ± 0.03; R4496C-TAC: 1.025 ± 0.05).

A



B

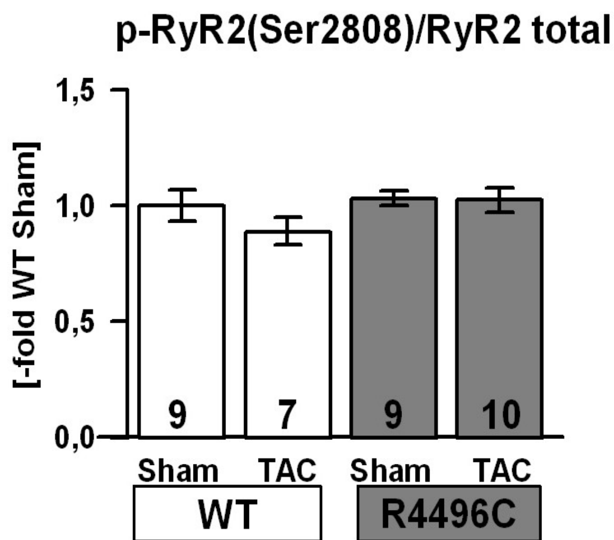


Figure 12: RyR2 phosphorylation level at residue Ser2808
A: Original Western Blot.
B: Mean values of fractional RyR2 phosphorylation at Ser2808. Data were normalized to WT-sham. Numbers in the bars indicate the number of hearts investigated.

3.1.2 RyR2 phosphorylation at Ser2814

Next, we studied RyR2 phosphorylation at CaMKII-dependent site Ser2814. Again, using a phosphorylation site-specific antibody, results were normalized to the total RyR2 protein expression. We detected no significant changes in fractional phosphorylation at residue serine 2814 (WT-sham: 1.000 ± 0.06; WT-TAC: 0.8935 ± 0.04; R4496C-sham: 0.8133 ± 0.20; R4496C-TAC: 0.8419 ± 0.06).

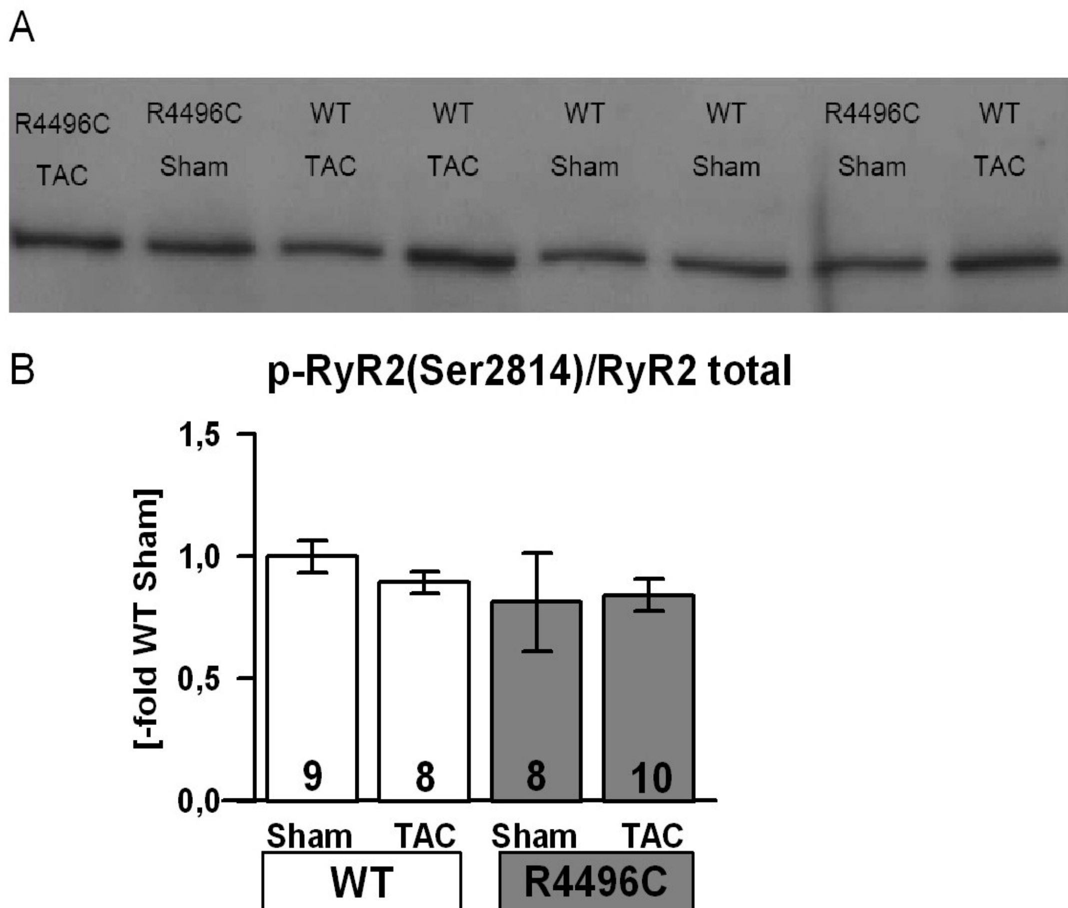
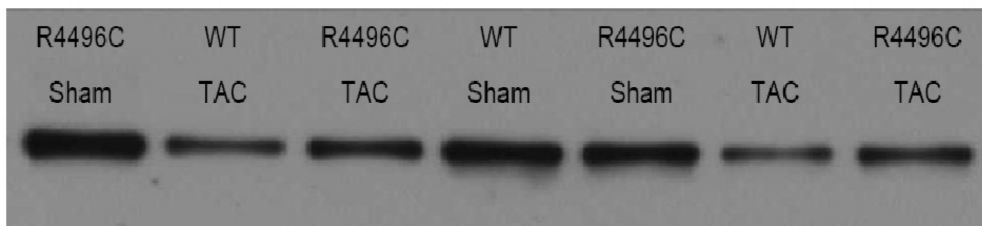


Figure 13: RyR2 phosphorylation level at residue Ser2814
A.: Original Western Blot.
B: Means of fractional phosphorylation status of RyR2 at Ser2814 normalized to the total RyR2 protein level. Data were normalized to WT-sham.

3.2 SERCA2a

The expression of SERCA2a was comparable in Sham-operated animals in both groups (WT-sham: 1.000 ± 0.10; R4496C-sham: 0.957 ± 0.12). In WT-TAC mice, SERCA2a expression was significantly reduced (0.674 ± 0.12) as compared to WT-sham. In R4496C-TAC mice, SERCA2a expression was further decreased (0.467 ± 0.05), although this did not reach statistical significance when compared with WT-TAC.

A



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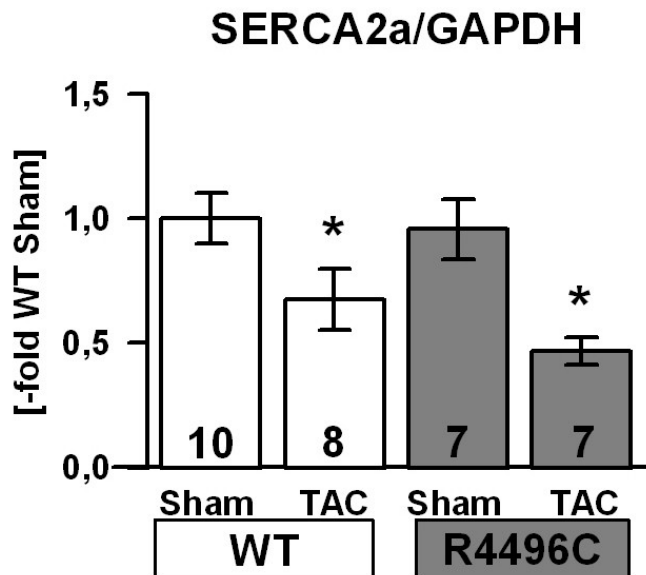


Figure 14: SERCA2a expression
A: Original Western Blot.
B: Average values of SERCA2a protein expression normalized to GAPDH. Data were normalized to WT-sham. Note reduced SERCA2a protein levels in both TAC groups versus respective Sham. *P < 0.05 versus respective Sham.

3.3 PLB

3.3.1 PLB total

One week after surgery, expression of PLB remained unchanged between all four groups (WT-sham: 1.000 ± 0.10; WT-TAC: 1.043 ± 0.10; R4496C-sham: 1.048 ± 0.16; R4496C-TAC: 0.895 ± 0.16).

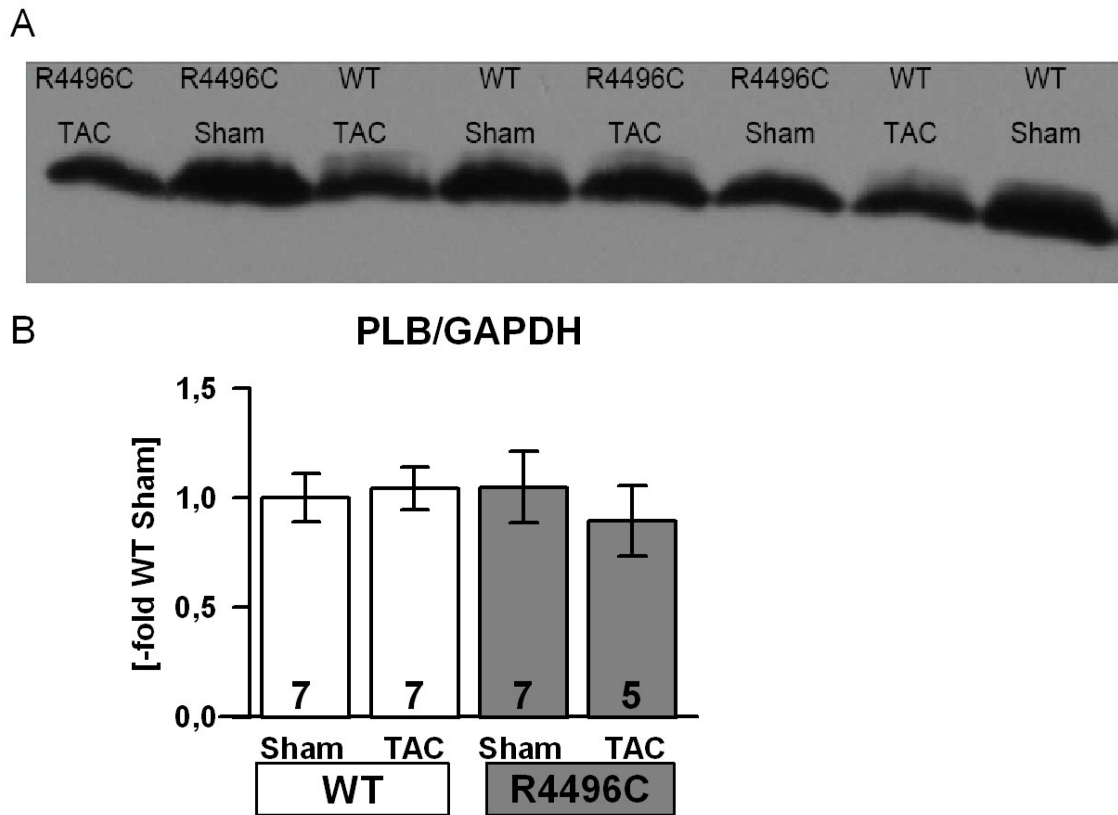
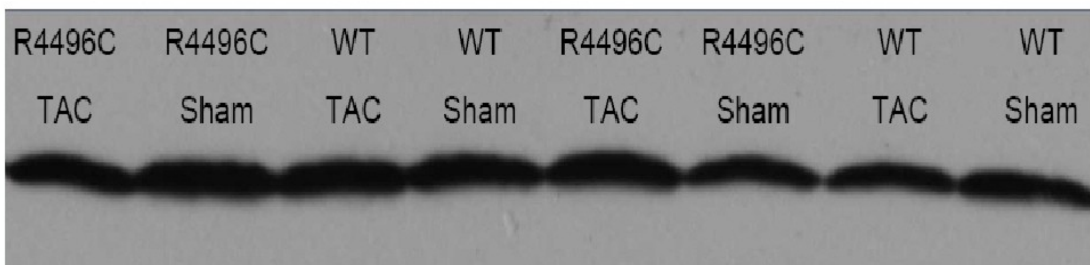


Figure 15: PLB expression
A: Original Western Blot.
B: Mean values of total PLB protein content, normalized to GAPDH. Data were normalized to WT-sham. No significant alterations occurred.

3.3.2 PLB phosphorylation level at Ser16

Phosphorylation site-specific antibodies were used to quantify phosphorylation status of PLB at the PKA-dependent site Ser16. Fractional phosphorylation was determined by normalization to the total PLB amount within the same sample. No significant differences in phosphorylation status were observed (WT-sham: 1.000 ± 010; WT-TAC: 0.872 ± 0.16; R4496C-sham: 0.971 ± 0.13; R4496C-TAC: 0.982 ± 0.16).

A



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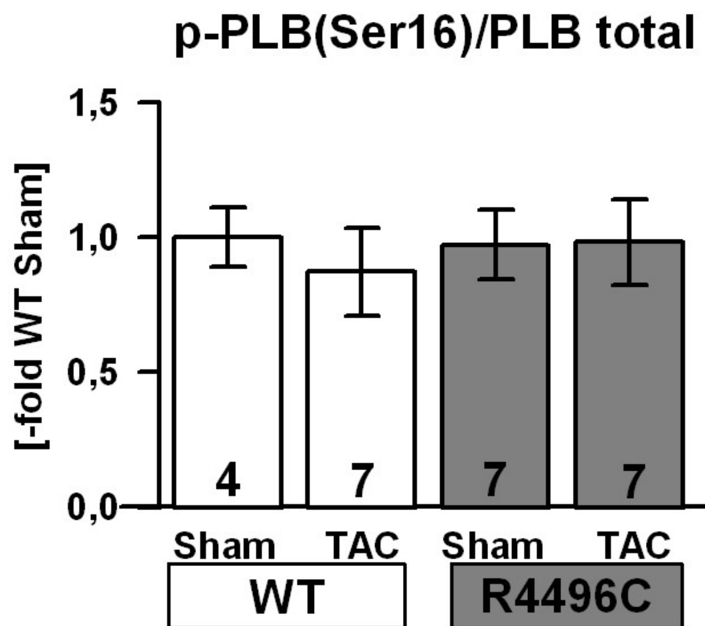


Figure 16: PLB phosphorylation at residue serine 16
A: Original Western Blot.
B: Means of fractional PLB phosphorylation status at Ser16. Results were normalized to WT-sham group.

3.3.3 PLB phosphorylation at Thr17

R4496C mice showed significantly reduced PLB phosphorylation at CamKII-dependent site Thr17, which was 58% and 46% of the WT hearts in Sham- and TAC-operated hearts, respectively. (WT-sham: 1.000 ± 0.10 versus R4496C-sham: 0.414 ± 0.08 and R4496C-TAC: 0.542 ± 0.06 versus WT-TAC: 0.846 ± 0.10, both $P<0.05$). No statistically significant differences were detected within the genotypes.

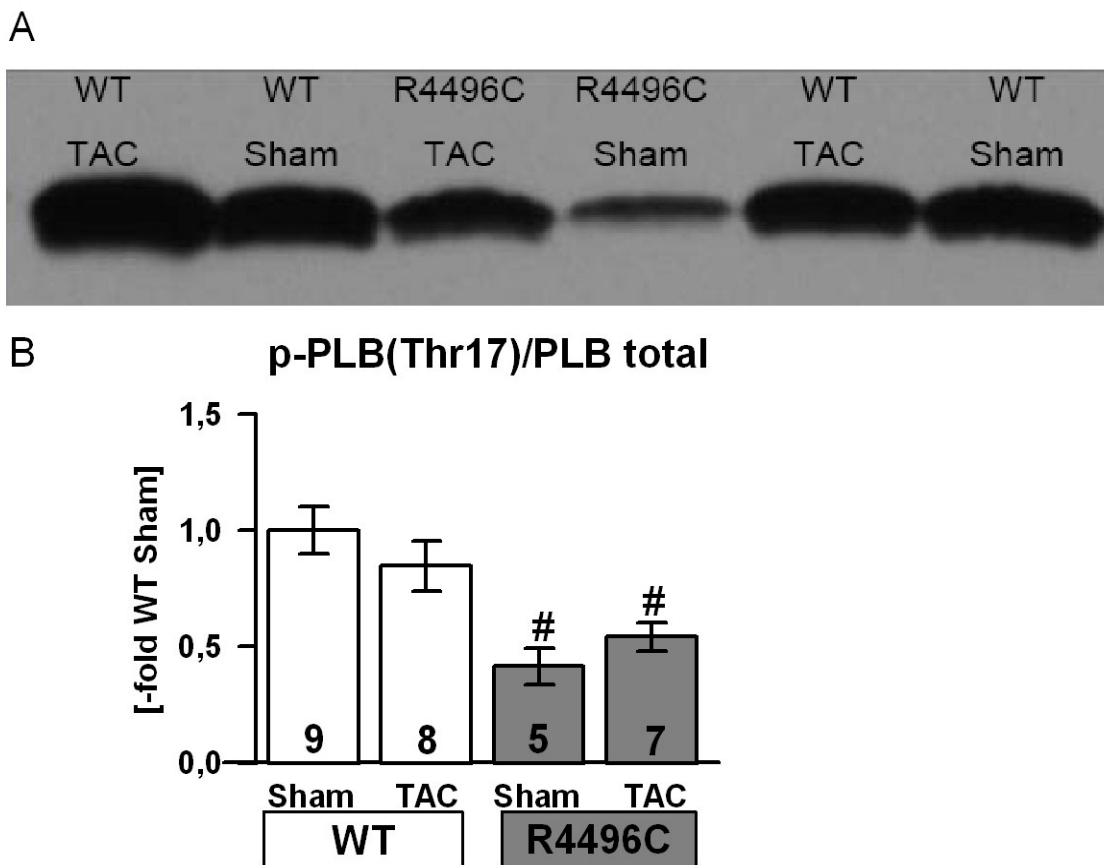
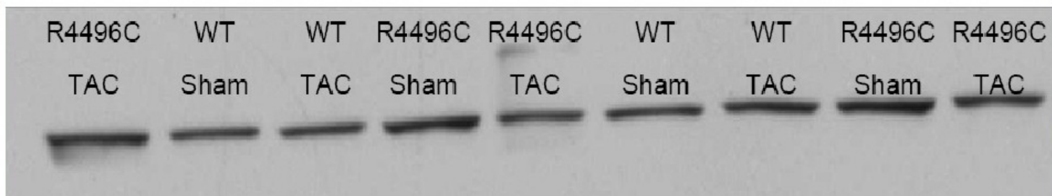


Figure 17: PLB phosphorylation at residue threonine 17
A: Original Western Blot.
B: Bar graph shows fractional phosphorylation at Thr17 normalized to the total PLB protein amount. Data were normalized to WT-sham group.
# P< 0.05 versus respective surgical intervention in WT

3.4 Calsequestrin

No statistical significant difference in CASQ2 expression was detected between the groups (WT-sham: 1.000 ± 0.14; WT-TAC: 0.901 ± 0.12; R4496C-sham: 1.034 ± 0.05; R4496C-TAC: 0.906 ± 0.13).

A



B

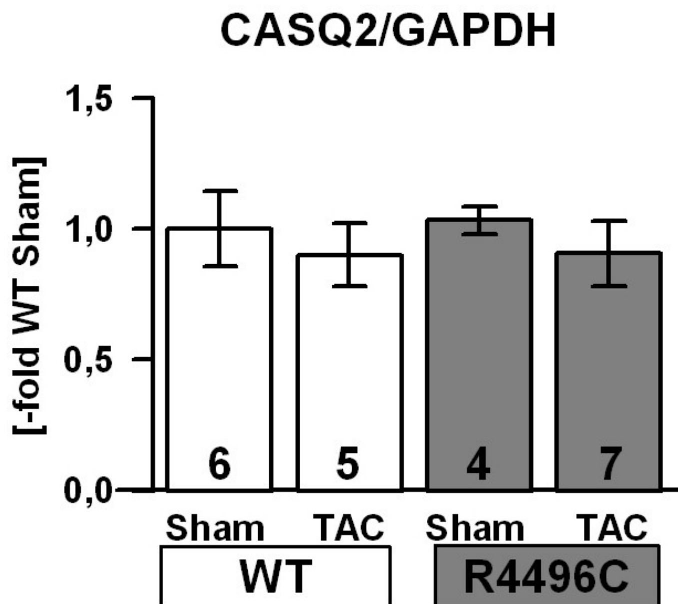


Figure 18: Calsequestrin expression
A: Original Western Blot.
B: CASQ2 protein expression was normalized to GAPDH and WT-sham.

4. Discussion

In the present study, we quantified the expression and phosphorylation of Ca^{2+} handling proteins, including RyR2, PLB, SERCA2a and CASQ2 in $RyR2^{R4496C+/-}$ murine cardiomyocytes after pressure overload-induced hypertrophy.
Cardiac myocytes from failing hearts show increased diastolic SR Ca^{2+} leak due to an enhanced RyR2 open probability, leading to alterations in intracellular Ca^{2+} homeostasis and reduced SR Ca^{2+} content, ultimately resulting in the genesis of arrhythmias and HF (Kushnir, Marks 2010). Other mechanisms contributing to the $[Ca^{2+}]_{SR}$ reduction are decreased SERCA2a function, due to its lower expression and increased inhibition by PLB, as well as an enhanced NCX expression, extruding more Ca^{2+} out of the cell (Bers 2006).

4.1 TAC

Transverse aortic constriction is a method used to induce LVH and HF in rodents (e.g. mice, rats). Aortic banding mimics human aortic stenosis, leading to hemodynamic overload and hypertrophy. While compensated in the beginning, the hypertrophy gradually deteriorates, ultimately resulting in cardiac dilation and overt HF. At early stages, compensation can even result in enhanced contractility. In contrast to other models of HF, such as HF induced by myocardial infarction (MI), the gradual time course of HF development is the main advantage of TAC (deAlmeida, van Oort & Wehrens 2010).
Recently, a CPVT-associated RyR2 mutation ($RyR2^{R176+/-}$) in mice subjected to TAC was shown to result in a pronounced hypertrophic response and faster progression of contractile failure, due to an increased diastolic SR Ca^{2+} leak, which induced a pro-hypertrophic Ca^{2+}-Cn-NFAT signaling pathway (van Oort et al. 2010). In agreement with that did we show that R4496C-TAC mice developed typical hallmarks of HF, such as eccentric hypertrophy and left ventricular dilation as early as one week post TAC. In contrast, WT-TAC mice exhibited concentric hypertrophy and did not develop left ventricular dilation one week after TAC (Sedej

et al., unpublished data). This suggests an accelerated progression of hypertrophy to HF in our R4496C-TAC model compared to WT-TAC mice.

4.2 RyR2-R4496C knock-in mice – the first CPVT model

The first family identified with CPVT, suffering from a highly malignant form resistant to β-blockers, was harboring a RyR2-R4497C mutation. This mutation, in which arginine at position 4497 is replaced by cysteine, was later identified in other CPVT patients as well. Thereafter, a heterozygous knock-in mouse model carrying a R4496C mutation, equivalent to the human R4497C mutation, was generated (Cerrone et al. 2005). At baseline, R4496C mice do not show phenotypical abnormalities, presenting with a normal life-span, cardiac structure and function. When exposed to β-adrenergic stimulation, however, bidirectional VTs are observed (Liu et al. 2006). An increased diastolic SR Ca^{2+} leak and enhanced Ca^{2+} spark frequency, suggesting elevated Ca^{2+} sensitivity of RyR2, at any given $[Ca^{2+}]_i$ was observed. A conformational change due to the highly active cysteine, introduced by the mutation, located closely to the site involved in Ca^{2+}-dependent activation, has been proposed to be causally involved in the arrhythmia development (Fernandez-Velasco et al. 2009). In the same mouse model, DADs appeared in unstimulated myocytes, but less so in WT cells, further supporting the hypothesis of increased RyR2 Ca^{2+} sensitivity (Liu et al. 2006). Reduced SR Ca^{2+} release threshold decreases SR Ca^{2+} content, resulting in diminished SR Ca^{2+} spark frequency. Elevation of SR Ca^{2+} load, however, elicited by β-adrenergic stimulation or ouabain, caused an increased Ca^{2+} spark frequency, increasing the propensity for arrhythmias (Sedej et al. 2010, Kashimura et al. 2010).

4.3 Ryanodine receptor

In failing human myocardium, numerous studies consistently observed unchanged levels of RyR2 expression (Meyer et al. 1995, Schillinger et al. 1996, Sainte Beuve et al. 1997). In animal models of HF, however, results on the expression levels of RyR2 are less consistent.

RyR2 expression levels were significantly decreased, while their Ca^{2+} sensitivity was highly increased, causing increased Ca^{2+} spark frequency and decreased $[Ca^{2+}]_{SR}$ in tachypacing-induced HF in dogs (Kubalova et al. 2005) but see also (Jiang et al. 2002). In rats, however, MI failed to induce changes in RyR2 expression on the mRNA as well as the protein level (Hu et al. 2011). Similarly, no alterations in RyR2 protein expression or phosphorylation were found in R4496C mice (Fernandez-Velasco et al. 2009).

4.3.1 PKA-dependent RyR2 phosphorylation

In HF, increased diastolic SR Ca^{2+} leak is caused by enhanced RyR2 open probability. Open probability of the RyR2 can be altered by channel phosphorylation, oxidation, S-nitrosylation or by mutations leading to activation of the channel due to higher sensitivity to Ca^{2+} (Kushnir, Marks 2010). RyR2 mutations have been found to appear mainly in three functionally important regions of the channel: (1) the binding site for the FKBP12.6 protein, (2) the channel-forming transmembrane domains and (3) the Ca^{2+}-binding site (Priori et al. 2002).

Marks' group (Marx et al. 2000) was the first to demonstrate an enhanced RyR2 open probability induced by PKA-dependent phosphorylation at Ser2808 in human and canine failing hearts, which, in turn, caused FKBP12.6 dissociation from the channel. This hypothesis was supported by a mouse model, harboring a RyR2 single point mutation S2808A, which prevents PKA-dependent phosphorylation at this site. The mutation protected mice from cardiac dysfunction and FKBP12.6 dissociation, observed in WT mice 4 weeks after MI (Wehrens et al. 2006). In the same mouse model, a blunted response to catecholaminergic stimulation was shown, resulting in reduced exercise capacity but protection from chronic catecholamine-induced cardiac dysfunction (Shan et al. 2010).

However, in the same canine model of tachycardia-induced HF as used by Marks' group, no difference in RyR2 Ca^{2+} sensitivity was shown by Jiang et al., using RyR2 reconstituted into planar lipid bilayers. Furthermore did PKA-dependent phosphorylation not dissociate FKBP12.6 from the channel. Because RyR2s were functionally and structurally normal, abnormal Ca^{2+} transients were explained by depressed SR function due to SERCA2a downregulation rather than RyR2 alteration (Jiang et al. 2002).

In our experiments, we found no alteration in PKA-dependent phosphorylation of RyR2 at Ser2808 (Fig. 12), neither after TAC nor due to the mutation. Consistently, no changes in phosphorylation of RyR2 at Ser2808 were reported in different animal models of HF (Kushnir, Marks 2010, Yeh et al. 2008). Our results were obtained as early as one week post TAC, therefore not representing terminal HF. In line with our findings, Ling et al., also investigating mice shortly after TAC, did not observe changes in PKA-dependent RyR2 phosphorylation two and six weeks after the intervention (Ling et al. 2009).

4.3.2 CaMKII-dependent RyR2 phosphorylation

RyR2 is also phosphorylated at Ser2814 by CaMKII, which is activated by increased intracellular Ca^{2+} concentration, as seen during β-adrenergic stimulation (Kushnir, Marks 2010).

TAC induced a significant increase in CaMKII-dependent RyR2 phosphorylation after six weeks (Ling et al. 2009), an increase in CaMKII signaling (Toischer et al. 2010) as well as CaMKII protein expression and autophosphorylation, indicating increased activity (Colomer et al. 2003) as early as one week after the procedure. Other groups, however, reported unchanged levels of CaMKII-dependent RyR2 phoshorylation 4 weeks post TAC (van Oort et al. 2010) or reduced CaMKII-dependent RyR2 and PLB phosphorylation with decreased levels of CaMKII expression and activity (Netticadan et al. 2000). Several reasons may explain these discrepancies, including different animal strains and species, different grades of stenosis induced by TAC, different experimental conditions, increased global expression of phosphatases as well as different stages of HF.

One week post TAC found our group no alterations in CaMKII-dependent phosphorylation of RyR2 at Ser2814 (Fig. 13). Consistent with this, showed electrically paced R4496C ventricular myocytes an increase in Ca^{2+} spark

frequency with no alterations in total or phosphorylated RyR2 levels (Fernandez-Velasco et al. 2009), but CaMKII inhibition prevented catecholamine-induced arrhythmias in R4496C mice (Liu et al. 2011).
Selective effects of CaMKII on RyR2 phosphorylation at Ser2814 were tested in a RyR2-S2814D mouse model, which mimics constitutive phosphorylation at the CaMKII-dependent phosphorylation site. RyR2-S2814D mutants showed increased sensitivity to catecholaminergic provocation, causing VT and sudden cardiac death. On the other hand, S2814A mice with a non-phosphorylatable residue were protected from arrhythmias. To further investigate the CaMKII-dependent effects of RyR2 phosphorylation in HF, RyR2-S2814D mice underwent TAC procedure, resulting in increased sudden arrhythmogenic death within the first three weeks in RyR2-S2814D mice compared to WT mice, suggesting that CaMKII-dependent RyR2 phosphorylation plays an important role in arrhythmogenesis in HF (van Oort et al. 2010).

4.4 SERCA2a

Decreased SERCA2a expression has been a consistent finding in previous studies using animal models with pressure overload-induced HF (Matsui et al. 1995, Aoyagi et al. 1999, Ling et al. 2009). These findings are supported by studies on failing human heart (Meyer et al. 1995, Schillinger et al. 1996, DiPaola et al. 2001). While a reduction of SERCA2a mRNA occurred in hypertrophied and failing human myocardium, a SERCA2a protein reduction was only observed in the failing heart group, suggesting a causal relation between the SERCA2a protein reduction and the development of HF (DiPaola et al. 2001).
On the other hand, no differences in SERCA2a and PLB protein expression were reported in patients with end-stage HF (Schmidt et al. 1999), but decreased PLB phosphorylation caused a significant reduction in SERCA2a activity.
In our experiments, we found a significant reduction in SERCA2a protein levels in mice one week after TAC, with a trend towards lower SERCA2a levels in mutant mice, which was, however, not statistically significant (Fig. 14). Reduced SERCA2a content results in decreased SR Ca^{2+} reuptake and lower $[Ca^{2+}]_{SR}$,

causing weaker contraction and slower relaxation (Bers 2006). While WT-TAC mice showed reduced SERCA2a expression levels as early as one week after the intervention, no reduction in contractility was observed at that point, suggesting that this degree of SERCA2a reduction is not sufficient to induce contractile failure. Unchanged expression levels of PLB (Fig. 15) and decreased PLB phosphorylation in R4496C mice (Fig. 17), further decreasing SERCA2a activity, however, may explain accelerated HF progression in mutant mice.

The impact of SERCA2a alterations on cardiac function has been investigated in genetically altered animal models. SERCA2a$^{+/-}$ mice have reduced SERCA2a protein levels and SR Ca^{2+} uptake. SERCA2a overexpression, on the other hand, leads to enhanced contractile function with no pathologies, and despite high mRNA levels, protein increase is only moderate, due to powerful post-transcriptional mechanisms (Periasamy, Huke 2001). In rats undergoing TAC, SERCA2a protein decreases in failing hearts but SERCA2a gene transfer is able to restore protein levels (Sakata et al. 2007). Adenoviral gene therapy also shows antiarrhythmic effects in rats with chronic HF, regarding spontaneous as well as catecholamine-induced arrhythmias. The gene therapy further leads to decreased SR Ca^{2+} leak and RyR2 dephosphorylation (Lyon et al. 2011). To evaluate gene transfer effects on humans, SERCA2a overexpression was induced in failing human myocardium, leading to faster contraction and relaxation velocity as well as an increase in systolic and a decrease in diastolic Ca^{2+} concentration (delMonte et al. 1999). Together, this indicates a crucial role of SERCA2a in altered Ca^{2+} cycling, leading to the development of HF.

4.5 Phospholamban

Normal PLB expression levels (Fig. 15) in conjunction with a SERCA2a decrease in the R4496C-TAC mice result in an increased PLB:SERCA2a ratio, implying greater SERCA2a inhibition, further aggravating typical HF signs caused by decreased SERCA2a activity.

Previous studies have reported a reduction in total PLB and SERCA2a expression in guinea-pig hearts after TAC (Kiss et al. 1995), while in a different animal model,

dogs with tachycardia-induced HF, no reduction of PLB and SERCA2a mRNA levels was shown (Williams et al. 1994).

In failing human hearts, a reduction in both proteins, with an increased ratio of PLB to SERCA2a, indicating lower pump activity (Hasenfuss et al. 1997), no change in PLB and SERCA2a expression (Movsesian et al. 1994), unchanged PLB with reduced SERCA2a expression (Dash et al. 2001), or unchanged levels of both proteins but reduced PLB phosphorylation, causing reduced SERCA2a activity (Schmidt et al. 1999), were reported. These inconsistencies might be due to individual variances, different causes and stages of HF as well as differences in previous therapy applied.

4.5.1 Phosphorylation of PLB

Numerous studies have provided inconsistent results with respect to the phosphorylation of PLB. A significant reduction in phosphorylation at Ser16 as well as Thr17 with unchanged total PLB expression was shown in failing human heart (Hu et al. 2011) and rats after MI (Dash et al. 2001). Whereas other studies showed either a significant decrease of phosphorylation at Thr17 with unaltered Ser16 phosphorylation status (Netticadan et al. 2000) or reduced phosphorylation levels at Ser16 with no change in Thr17 phosphorylation (Sande et al. 2002) in the same HF model. A significant increase in phosphatase 1 (PP1) and 2 (PP2) in both groups, with reduced endogenous CaMKII content as well as an overall reduction in SR proteins RyR2, SERCA2a and PLB (Netticadan et al. 2000) or no alteration in PKA regulatory subunit II as well as RyR2 and SERCA2a (Sande et al. 2002) was further shown.

In the present study, we found no alterations in total PLB protein expression (Fig. 15) or PLB phosphorylation at Ser16 (Fig. 16). We did, however, observe a significant reduction in PLB phosphorylation levels at Thr17 in R4496C mice (Fig. 17), further reducing SERCA2a activity. Because dephosphorylation occurred in R4496C mice independently from the intervention, other contributing factors need to be considered. Calcineurin, a serine-threonine phosphatase, is activated by sustained elevations in intracellular Ca^{2+} (Gelpi et al. 2009), as seen in R4496C mice. It plays a role as a sensing molecule, mediating a cardiac hypertrophic response by activating cardiac transcription factors (Gelpi et al. 2009). $R176Q^{+/-}$

mice, harboring another CPVT associated mutation, showed increased Cn expression levels after TAC, suggesting the increased SR Ca^{2+} leak to activate a prohypertrophic Cn-NFAT pathway (van Oort et al. 2010). Further investigations showed that transgenic overexpression of the constitutively active Cn form led to a dramatic increase in heart size and hypertrophy, findings consistent with HF, and made the animals more susceptible to sudden cardiac death (Molkentin et al. 1998). Transgenic mice, overexpressing a Cn inhibitor, on the other hand, showed less LVH two weeks after aortic banding and decreased PLB dephosphorylation (Gelpi et al. 2009). In rabbits with a complete AV-block and implanted ICD, an increase in CaMKII autophosphorylation, indicating higher activity, was seen. This was accompanied by a significant PLB dephosphorylation, for which an increase in PP1 and PP2A was made accountable for (Tsuji et al. 2011). Increased levels of PP1 and PP2A were further shown in another HF model (Netticadan et al. 2000, Sande al. 2002). All of these phosphatases can contribute to PLB dephosphorylation, further impairing SERCA2a function.

4.6 Calsequestrin

In the present study, no alteration in CASQ2 expression was found one week after TAC (Fig. 18), suggesting no important role of CASQ2 in HF development. Similar results were obtained in human failing myocardium (Movsesian et al. 1994, Meyer et al. 1995, Schillinger et al. 1996).

A significant reduction in CASQ2 protein and mRNA expression was shown in rats after MI (Hu et al. 2011), but a decrease in almost all proteins investigated (PP1, PP2, SERCA2a, PLB-Thr17, PLB-Ser16, sorcin, CaM) suggests an overall decrease in protein quantity in this model.

To further elucidate the function of CASQ2, genetically altered mice were investigated. Transgenic mice overexpressing CASQ2 showed depressed fractional SR Ca^{2+} release due to higher SR Ca^{2+} buffering. This process induced hypertrophy signaling implicated in the HF development (Gregory, Kranias 2006). In contrast, complete deletion of the protein did not cause reduction in SR Ca^{2+}

content, but was compensated by a dramatic SR volume increase and downregulation of triadin and junctin. Mutant mice showed normal cardiac function under baseline conditions and increased SR Ca^{2+} leak with an increased risk of triggered arrhythmias when exposed to catecholamines (Knollmann et al. 2006). Other studies further showed unaltered levels of CASQ2 expression in animal models of HF (Engelhardt et al. 2001, Fan et al. 2004).

4.7 Conclusion and Perspectives

The major finding of my diploma thesis was reduced SERCA2a expression in conjunction with reduced phosphorylation of PLB at Thr17 in R4496C-TAC mice, implying reduced SERCA2a activity in this model early after TAC. This suggests an impaired SR Ca^{2+} reuptake and, thus, reduced SR Ca^{2+} content underlying the contractile dysfunction (reduced contraction and slower relaxation). This finding helps to explain – at least partially – the rapid development of overt HF in R4496C mice imposed to pressure overload. Furthermore, RyR2 phosphorylation at both major phosphorylation sites, Ser2808 and Ser2814, as well as PLB phosphorylation at Ser16 was unchanged one week after TAC.
The future quantification of the expression and activity of major modulators of Ca^{2+}-dependent proteins, including phosphatases and kinases, such as PKA and CaMKII, will give a better insight to the regulation of Ca^{2+}-handling proteins in our R4496C-TAC model.

Literature

Ai, X., Curran, J.W., Shannon, T.R., Bers, D.M. & Pogwizd, S.M. 2005, "Ca2+/calmodulin-dependent protein kinase modulates cardiac ryanodine receptor phosphorylation and sarcoplasmic reticulum Ca2+ leak in heart failure", *Circulation research,* vol. 97, no. 12, pp. 1314-1322.

Aoyagi, T., Yonekura, K., Eto, Y., Matsumoto, A., Yokoyama, I., Sugiura, S., Momomura, S., Hirata, Y., Baker, D.L. & Periasamy, M. 1999, "The sarcoplasmic reticulum Ca2+-ATPase (SERCA2) gene promoter activity is decreased in response to severe left ventricular pressure-overload hypertrophy in rat hearts", *Journal of Molecular and Cellular Cardiology,* vol. 31, no. 4, pp. 919-926.

Bassani, R.A., Bassani, J.W. & Bers, D.M. 1992, "Mitochondrial and sarcolemmal Ca2+ transport reduce [Ca2+]i during caffeine contractures in rabbit cardiac myocytes", *The Journal of physiology,* vol. 453, pp. 591-608.

Bers, D.M. 2001, *Excitation-contraction coupling and cardiac contractile force,* Kluwer Academic Press.

Bers, D.M. 2008, "Calcium cycling and signaling in cardiac myocytes", *Annual Review of Physiology,* vol. 70, pp. 23-49.

Bers, D.M. 2006, "Altered cardiac myocyte Ca regulation in heart failure", *Physiology (Bethesda, Md.),* vol. 21, pp. 380-387.

Bers, D.M. 2002, "Cardiac excitation-contraction coupling", *Nature,* vol. 415, no. 6868, pp. 198-205.

Bers, D.M. & Guo, T. 2005, "Calcium signaling in cardiac ventricular myocytes", *Annals of the New York Academy of Sciences,* vol. 1047, pp. 86-98.

Blayney, L.M. & Lai, F.A. *Ryanodine receptor-mediated arrhythmias and sudden cardiac death*.

Blayney, L.M. & Lai, F.A. 2009, "Ryanodine receptor-mediated arrhythmias and sudden cardiac death", *Pharmacology & therapeutics,* vol. 123, no. 2, pp. 151-177.

Cerrone, M., Colombi, B., Santoro, M., di Barletta, M.R., Scelsi, M., Villani, L., Napolitano, C. & Priori, S.G. 2005, "Bidirectional ventricular tachycardia and fibrillation elicited in a knock-in mouse model carrier of a mutation in the cardiac ryanodine receptor", *Circulation research,* vol. 96, no. 10, pp. e77-82.

Cerrone, M., Napolitano, C. & Priori, S.G. 2009, "Catecholaminergic polymorphic ventricular tachycardia: A paradigm to understand mechanisms of arrhythmias associated to impaired Ca(2+) regulation", *Heart rhythm : the official journal of the Heart Rhythm Society,* vol. 6, no. 11, pp. 1652-1659.

Colomer, J.M., Mao, L., Rockman, H.A. & Means, A.R. 2003, "Pressure overload selectively up-regulates Ca2+/calmodulin-dependent protein kinase II in vivo", *Molecular endocrinology (Baltimore, Md.),* vol. 17, no. 2, pp. 183-192.

Cowie, M.R., Wood, D.A., Coats, A.J., Thompson, S.G., Poole-Wilson, P.A., Suresh, V. & Sutton, G.C. 1999, "Incidence and aetiology of heart failure; a population-based study", *European heart journal,* vol. 20, no. 6, pp. 421-428.

Dash, R., Frank, K.F., Carr, A.N., Moravec, C.S. & Kranias, E.G. 2001, "Gender influences on sarcoplasmic reticulum Ca2+-handling in failing human myocardium", *Journal of Molecular and Cellular Cardiology,* vol. 33, no. 7, pp. 1345-1353.

deAlmeida, A.C., van Oort, R.J. & Wehrens, X.H. 2010, "Transverse aortic constriction in mice", *Journal of visualized experiments : JoVE,* vol. (38). pii: 1729. doi, no. 38, pp. 10.3791/1729.

del, M., Harding, S.E., Schmidt, U., Matsui, T., Kang, Z.B., Dec, G.W., Gwathmey, J.K., Rosenzweig, A. & Hajjar, R.J. 1999, "Restoration of contractile function in isolated cardiomyocytes from failing human hearts by gene transfer of SERCA2a", *Circulation,* vol. 100, no. 23, pp. 2308-2311.

DiPaola, N.R., Sweet, W.E., Stull, L.B., Francis, G.S. & Schomisch Moravec, C. 2001, "Beta-adrenergic receptors and calcium cycling proteins in non-failing, hypertrophied and failing human hearts: transition from hypertrophy to failure", *Journal of Molecular and Cellular Cardiology,* vol. 33, no. 6, pp. 1283-1295.

Duan, D.D. 2010, "A leakage leads to failure: roles of sarcoplasmic reticulum Ca2+ leak via RyR2 in heart failure progression", *Hypertension,* vol. 55, no. 4, pp. 849-851.

Endo, M. 2009, "Calcium-induced calcium release in skeletal muscle", *Physiological Reviews,* vol. 89, no. 4, pp. 1153-1176.

Engelhardt, S., Boknik, P., Keller, U., Neumann, J., Lohse, M.J. & Hein, L. 2001, "Early impairment of calcium handling and altered expression of junctin in hearts of mice overexpressing the beta1-adrenergic receptor", *The FASEB journal : official publication of the Federation of American Societies for Experimental Biology,* vol. 15, no. 14, pp. 2718-2720.

Erdmann, E. (ed) 2011, *Klinische Kardiologie*, 8th edn, Springer Medizin Verlag Heidelberg.

Erkasap, N. 2007, "SERCA in genesis of arrhythmias: what we already know and what is new?", *Anadolu kardiyoloji dergisi : AKD = the Anatolian journal of cardiology,* vol. 7 Suppl 1, pp. 43-46.

Fan, G.C., Gregory, K.N., Zhao, W., Park, W.J. & Kranias, E.G. 2004, "Regulation of myocardial function by histidine-rich, calcium-binding protein", *American journal of physiology.Heart and circulatory physiology,* vol. 287, no. 4, pp. H1705-11.

Fernandez-Velasco, M., Rueda, A., Rizzi, N., Benitah, J.P., Colombi, B., Napolitano, C., Priori, S.G., Richard, S. & Gomez, A.M. 2009, "Increased Ca2+ sensitivity of the ryanodine receptor mutant RyR2R4496C underlies catecholaminergic polymorphic ventricular tachycardia", *Circulation research,* vol. 104, no. 2, pp. 201-9, 12p following 209.

Gelpi, R.J., Gao, S., Zhai, P., Yan, L., Hong, C., Danridge, L.M., Ge, H., Maejima, Y., Donato, M., Yokota, M., Molkentin, J.D., Vatner, D.E., Vatner, S.F. & Sadoshima, J. 2009, "Genetic inhibition of calcineurin induces diastolic dysfunction in mice with chronic pressure overload", *American journal of physiology.Heart and circulatory physiology,* vol. 297, no. 5, pp. H1814-9.

Gregory, K.N. & Kranias, E.G. 2006, "Targeting sarcoplasmic reticulum calcium handling proteins as therapy for cardiac disease", *Hellenic journal of cardiology : HJC = Hellenike kardiologike epitheorese,* vol. 47, no. 3, pp. 132-143.

Grimm, W. & Maisch, B. 2002, "Sudden cardiac death in dilated cardiomyopathy -- therapeutic options", *Herz,* vol. 27, no. 8, pp. 750-759.

Gyorke, I., Hester, N., Jones, L.R. & Gyorke, S. 2004, "The role of calsequestrin, triadin, and junctin in conferring cardiac ryanodine receptor responsiveness to luminal calcium", *Biophysical journal,* vol. 86, no. 4, pp. 2121-2128.

Gyorke, S. 2009, "Molecular basis of catecholaminergic polymorphic ventricular tachycardia", *Heart rhythm : the official journal of the Heart Rhythm Society,* vol. 6, no. 1, pp. 123-129.

Gyorke, S. & Terentyev, D. 2008, "Modulation of ryanodine receptor by luminal calcium and accessory proteins in health and cardiac disease", *Cardiovascular research,* vol. 77, no. 2, pp. 245-255.

Hasenfuss, G., Meyer, M., Schillinger, W., Preuss, M., Pieske, B. & Just, H. 1997, "Calcium handling proteins in the failing human heart", *Basic research in cardiology,* vol. 92 Suppl 1, pp. 87-93.

Hasenfuss, G. & Pieske, B. 2002, "Calcium cycling in congestive heart failure", *Journal of Molecular and Cellular Cardiology,* vol. 34, no. 8, pp. 951-969.

Höllinger, K. 2011, *, Herzinsuffizienz*. Available: http://www.turnusdoc.at/media/weblog/Herzinsuffizienz.ppt [2011, .

Hu, P., Zhang, D., Swenson, L., Chakrabarti, G., Abel, E.D. & Litwin, S.E. 2003, "Minimally invasive aortic banding in mice: effects of altered cardiomyocyte insulin signaling during pressure overload", *American journal of physiology.Heart and circulatory physiology,* vol. 285, no. 3, pp. H1261-9.

Hu, S.T., Liu, G.S., Shen, Y.F., Wang, Y.L., Tang, Y. & Yang, Y.J. 2011, "Defective Ca(2+) handling proteins regulation during heart failure", *Physiological Research / Academia Scientiarum Bohemoslovaca,* vol. 60, no. 1, pp. 27-37.

Ikemoto, N. & Yamamoto, T. 2002, "Regulation of calcium release by interdomain interaction within ryanodine receptors", *Frontiers in bioscience : a journal and virtual library,* vol. 7, pp. d671-83.

Jiang, D., Wang, R., Xiao, B., Kong, H., Hunt, D.J., Choi, P., Zhang, L. & Chen, S.R. 2005, "Enhanced store overload-induced Ca2+ release and channel sensitivity to luminal Ca2+ activation are common defects of RyR2 mutations linked to ventricular tachycardia and sudden death", *Circulation research,* vol. 97, no. 11, pp. 1173-1181.

Jiang, M.T., Lokuta, A.J., Farrell, E.F., Wolff, M.R., Haworth, R.A. & Valdivia, H.H. 2002, "Abnormal Ca2+ release, but normal ryanodine receptors, in canine and human heart failure", *Circulation research,* vol. 91, no. 11, pp. 1015-1022.

Kannankeril, P.J., Mitchell, B.M., Goonasekera, S.A., Chelu, M.G., Zhang, W., Sood, S., Kearney, D.L., Danila, C.I., De Biasi, M., Wehrens, X.H., Pautler, R.G., Roden, D.M., Taffet, G.E., Dirksen, R.T., Anderson, M.E. & Hamilton, S.L. 2006, "Mice with the R176Q cardiac ryanodine receptor mutation exhibit catecholamine-induced ventricular tachycardia and cardiomyopathy", *Proceedings of the National Academy of Sciences of the United States of America,* vol. 103, no. 32, pp. 12179-12184.

Kashimura, T., Briston, S.J., Trafford, A.W., Napolitano, C., Priori, S.G., Eisner, D.A. & Venetucci, L.A. 2010, "In the RyR2(R4496C) mouse model of CPVT, beta-adrenergic stimulation induces Ca waves by increasing SR Ca content and not by decreasing the threshold for Ca waves", *Circulation research,* vol. 107, no. 12, pp. 1483-1489.

Kiss, E., Ball, N.A., Kranias, E.G. & Walsh, R.A. 1995, "Differential changes in cardiac phospholamban and sarcoplasmic reticular Ca(2+)-ATPase protein levels. Effects on Ca2+ transport and mechanics in compensated pressure-overload hypertrophy and congestive heart failure", *Circulation research,* vol. 77, no. 4, pp. 759-764.

Knollmann, B.C., Chopra, N., Hlaing, T., Akin, B., Yang, T., Ettensohn, K., Knollmann, B.E., Horton, K.D., Weissman, N.J., Holinstat, I., Zhang, W., Roden, D.M., Jones, L.R., Franzini-Armstrong, C. & Pfeifer, K. 2006, "Casq2 deletion causes sarcoplasmic reticulum volume increase, premature Ca2+ release, and catecholaminergic polymorphic ventricular tachycardia", *The Journal of clinical investigation,* vol. 116, no. 9, pp. 2510-2520.

Kozanli., I., Jarai, R., Fellner, B., Jakl, G. & Huber, K. 2008, "Frauen und Herzinsuffizienz", *Austrian Journal of Cardiology,* vol. 15, pp. 292-297.

Kubalova, Z., Terentyev, D., Viatchenko-Karpinski, S., Nishijima, Y., Gyorke, I., Terentyeva, R., da Cunha, D.N., Sridhar, A., Feldman, D.S., Hamlin, R.L., Carnes, C.A. & Gyorke, S. 2005, "Abnormal intrastore calcium signaling in chronic heart failure", *Proceedings of the National Academy of Sciences of the United States of America,* vol. 102, no. 39, pp. 14104-14109.

Kushnir, A. & Marks, A.R. 2010, "The ryanodine receptor in cardiac physiology and disease", *Advances in Pharmacology (San Diego, Calif.),* vol. 59, pp. 1-30.

Lahat, H., Pras, E., Olender, T., Avidan, N., Ben-Asher, E., Man, O., Levy-Nissenbaum, E., Khoury, A., Lorber, A., Goldman, B., Lancet, D. & Eldar, M. 2001, "A missense mutation in a highly conserved region of CASQ2 is associated with autosomal recessive catecholamine-induced polymorphic ventricular tachycardia in Bedouin families from Israel", *American Journal of Human Genetics,* vol. 69, no. 6, pp. 1378-1384.

Lanner, J.T., Georgiou, D.K., Joshi, A.D. & Hamilton, S.L. 2010, "Ryanodine receptors: structure, expression, molecular details, and function in calcium release", *Cold Spring Harbor perspectives in biology,* vol. 2, no. 11, pp. a003996.

Leenhardt, A., Lucet, V., Denjoy, I., Grau, F., Ngoc, D.D. & Coumel, P. 1995, "Catecholaminergic polymorphic ventricular tachycardia in children. A 7-year follow-up of 21 patients", *Circulation,* vol. 91, no. 5, pp. 1512-1519.

Lehnart, S.E., Maier, L.S. & Hasenfuss, G. 2009, "Abnormalities of calcium metabolism and myocardial contractility depression in the failing heart", *Heart failure reviews,* vol. 14, no. 4, pp. 213-224.

Lehnart, S.E., Mongillo, M., Bellinger, A., Lindegger, N., Chen, B.X., Hsueh, W., Reiken, S., Wronska, A., Drew, L.J., Ward, C.W., Lederer, W.J., Kass, R.S., Morley, G. & Marks, A.R. 2008, "Leaky Ca2+ release channel/ryanodine receptor 2 causes seizures and sudden cardiac death in mice", *The Journal of clinical investigation,* vol. 118, no. 6, pp. 2230-2245.

Leszek, P., Szperl, M., Klisiewicz, A., Janas, J., Biederman, A., Rywik, T., Piotrowski, W., Kopacz, M. & Korewicki, J. 2007, "Alteration of myocardial sarcoplasmic reticulum Ca2+-ATPase and Na+-Ca2+ exchanger expression in human left ventricular volume overload", *European journal of heart failure,* vol. 9, no. 6-7, pp. 579-586.

Ling, H., Zhang, T., Pereira, L., Means, C.K., Cheng, H., Gu, Y., Dalton, N.D., Peterson, K.L., Chen, J., Bers, D. & Heller Brown, J. 2009, "Requirement for Ca2+/calmodulin-dependent kinase II in the transition from pressure overload-induced cardiac hypertrophy to heart failure in mice", *The Journal of clinical investigation,* vol. 119, no. 5, pp. 1230-1240.

Liu, N., Colombi, B., Memmi, M., Zissimopoulos, S., Rizzi, N., Negri, S., Imbriani, M., Napolitano, C., Lai, F.A. & Priori, S.G. 2006, "Arrhythmogenesis in catecholaminergic polymorphic ventricular tachycardia: insights from a RyR2 R4496C knock-in mouse model", *Circulation research,* vol. 99, no. 3, pp. 292-298.

Liu, N., Ruan, Y., Denegri, M., Bachetti, T., Li, Y., Colombi, B., Napolitano, C., Coetzee, W.A. & Priori, S.G. 2011, "Calmodulin kinase II inhibition prevents arrhythmias in RyR2(R4496C+/-) mice with catecholaminergic polymorphic ventricular tachycardia", *Journal of Molecular and Cellular Cardiology,* vol. 50, no. 1, pp. 214-222.

Liu, N., Ruan, Y. & Priori, S.G. 2008, "Catecholaminergic polymorphic ventricular tachycardia", *Progress in cardiovascular diseases,* vol. 51, no. 1, pp. 23-30.

Lyon, A.R., Bannister, M.L., Collins, T., Pearce, E., Sepehripour, A.H., Dubb, S.S., Garcia, E., O'Gara, P., Liang, L., Kohlbrenner, E., Hajjar, R.J., Peters, N.S., Poole-Wilson, P.A., Macleod, K.T. & Harding, S.E. 2011, "SERCA2a gene transfer decreases sarcoplasmic reticulum calcium leak and reduces ventricular arrhythmias in a model of chronic heart failure", *Circulation.Arrhythmia and electrophysiology,* vol. 4, no. 3, pp. 362-372.

MacLennan, D.H. & Kranias, E.G. 2003, "Phospholamban: a crucial regulator of cardiac contractility", *Nature reviews.Molecular cell biology,* vol. 4, no. 7, pp. 566-577.

Marx, S.O., Reiken, S., Hisamatsu, Y., Jayaraman, T., Burkhoff, D., Rosemblit, N. & Marks, A.R. 2000, "PKA phosphorylation dissociates FKBP12.6 from the calcium release channel (ryanodine receptor): defective regulation in failing hearts", *Cell,* vol. 101, no. 4, pp. 365-376.

Matsui, H., MacLennan, D.H., Alpert, N.R. & Periasamy, M. 1995, "Sarcoplasmic reticulum gene expression in pressure overload-induced cardiac hypertrophy in rabbit", *The American Journal of Physiology,* vol. 268, no. 1 Pt 1, pp. C252-8.

McMurray, J.J. & Stewart, S. 2000, "Epidemiology, aetiology, and prognosis of heart failure", *Heart (British Cardiac Society),* vol. 83, no. 5, pp. 596-602.

Meissner, G. & Henderson, J.S. 1987, "Rapid calcium release from cardiac sarcoplasmic reticulum vesicles is dependent on Ca2+ and is modulated by Mg2+, adenine nucleotide, and calmodulin", *The Journal of biological chemistry,* vol. 262, no. 7, pp. 3065-3073.

Meyer, M., Schillinger, W., Pieske, B., Holubarsch, C., Heilmann, C., Posival, H., Kuwajima, G., Mikoshiba, K., Just, H. & Hasenfuss, G. 1995, "Alterations of sarcoplasmic reticulum proteins in failing human dilated cardiomyopathy", *Circulation,* vol. 92, no. 4, pp. 778-784.

Minamisawa, S., Sato, Y. & Cho, M.C. 2004, "Calcium cycling proteins in heart failure, cardiomyopathy and arrhythmias", *Experimental & molecular medicine,* vol. 36, no. 3, pp. 193-203.

Mohamed, U., Napolitano, C. & Priori, S.G. 2007, "Molecular and electrophysiological bases of catecholaminergic polymorphic ventricular tachycardia", *Journal of cardiovascular electrophysiology,* vol. 18, no. 7, pp. 791-797.

Mohler, P.J. & Wehrens, X.H. 2007, "Mechanisms of human arrhythmia syndromes: abnormal cardiac macromolecular interactions", *Physiology (Bethesda, Md.),* vol. 22, pp. 342-350.

Molkentin, J.D., Lu, J.R., Antos, C.L., Markham, B., Richardson, J., Robbins, J., Grant, S.R. & Olson, E.N. 1998, "A calcineurin-dependent transcriptional pathway for cardiac hypertrophy", *Cell,* vol. 93, no. 2, pp. 215-228.

Movsesian, M.A., Karimi, M., Green, K. & Jones, L.R. 1994, "Ca(2+)-transporting ATPase, phospholamban, and calsequestrin levels in nonfailing and failing human myocardium", *Circulation,* vol. 90, no. 2, pp. 653-657.

Mudd, J.O. & Kass, D.A. 2008, "Tackling heart failure in the twenty-first century", *Nature,* vol. 451, no. 7181, pp. 919-928.

Netticadan, T., Temsah, R.M., Kawabata, K. & Dhalla, N.S. 2000, "Sarcoplasmic reticulum Ca(2+)/Calmodulin-dependent protein kinase is altered in heart failure", *Circulation research,* vol. 86, no. 5, pp. 596-605.

Oda, T., Yano, M., Yamamoto, T., Tokuhisa, T., Okuda, S., Doi, M., Ohkusa, T., Ikeda, Y., Kobayashi, S., Ikemoto, N. & Matsuzaki, M. 2005, "Defective regulation of interdomain interactions within the ryanodine receptor plays a key role in the pathogenesis of heart failure", *Circulation,* vol. 111, no. 25, pp. 3400-3410.

Pavlovic, M., Schaller, A., Pfammatter, J.P., Carrel, T., Berdat, P. & Gallati, S. 2005, "Age-dependent suppression of SERCA2a mRNA in pediatric atrial myocardium", *Biochemical and biophysical research communications,* vol. 326, no. 2, pp. 344-348.

Periasamy, M. & Huke, S. 2001, "SERCA pump level is a critical determinant of Ca(2+)homeostasis and cardiac contractility", *Journal of Molecular and Cellular Cardiology,* vol. 33, no. 6, pp. 1053-1063.

Pogwizd, S.M. & Bers, D.M. 2004, "Cellular basis of triggered arrhythmias in heart failure", *Trends in cardiovascular medicine,* vol. 14, no. 2, pp. 61-66.

Priori, S.G. & Chen, S.R. 2011, "Inherited dysfunction of sarcoplasmic reticulum Ca2+ handling and arrhythmogenesis", *Circulation research,* vol. 108, no. 7, pp. 871-883.

Priori, S.G., Napolitano, C., Memmi, M., Colombi, B., Drago, F., Gasparini, M., DeSimone, L., Coltorti, F., Bloise, R., Keegan, R., Cruz Filho, F.E., Vignati, G., Benatar, A. & DeLogu, A. 2002, "Clinical and molecular characterization of patients with catecholaminergic polymorphic ventricular tachycardia", *Circulation,* vol. 106, no. 1, pp. 69-74.

Priori, S.G., Napolitano, C., Tiso, N., Memmi, M., Vignati, G., Bloise, R., Sorrentino, V. & Danieli, G.A. 2001, "Mutations in the cardiac ryanodine receptor gene (hRyR2) underlie catecholaminergic polymorphic ventricular tachycardia", *Circulation,* vol. 103, no. 2, pp. 196-200.

Rickenbacher, P. 2001, "Herzinsuffizienz: Epidemiologie, Pathophysiologie", *Swiss Medical Forum,* , no. 1/2, pp. 4-9.

Rodriguez, P., Bhogal, M.S. & Colyer, J. 2003, "Stoichiometric phosphorylation of cardiac ryanodine receptor on serine 2809 by calmodulin-dependent kinase II and protein kinase A", *The Journal of biological chemistry,* vol. 278, no. 40, pp. 38593-38600.

Roger, V.L., Go, A.S., Lloyd-Jones, D.M., Adams, R.J., Berry, J.D., Brown, T.M., Carnethon, M.R., Dai, S., de Simone, G., Ford, E.S., Fox, C.S., Fullerton, H.J.,

Gillespie, C., Greenlund, K.J., Hailpern, S.M., Heit, J.A., Ho, P.M., Howard, V.J., Kissela, B.M., Kittner, S.J., Lackland, D.T., Lichtman, J.H., Lisabeth, L.D., Makuc, D.M., Marcus, G.M., Marelli, A., Matchar, D.B., McDermott, M.M., Meigs, J.B., Moy, C.S., Mozaffarian, D., Mussolino, M.E., Nichol, G., Paynter, N.P., Rosamond, W.D., Sorlie, P.D., Stafford, R.S., Turan, T.N., Turner, M.B., Wong, N.D., Wylie-Rosett, J. & American Heart Association Statistics Committee and Stroke Statistics Subcommittee 2011, "Heart disease and stroke statistics--2011 update: a report from the American Heart Association", *Circulation,* vol. 123, no. 4, pp. e18-e209.

Sainte Beuve, C., Allen, P.D., Dambrin, G., Rannou, F., Marty, I., Trouve, P., Bors, V., Pavie, A., Gandgjbakch, I. & Charlemagne, D. 1997, "Cardiac calcium release channel (ryanodine receptor) in control and cardiomyopathic human hearts: mRNA and protein contents are differentially regulated", *Journal of Molecular and Cellular Cardiology,* vol. 29, no. 4, pp. 1237-1246.

Sakata, S., Lebeche, D., Sakata, N., Sakata, Y., Chemaly, E.R., Liang, L.F., Tsuji, T., Takewa, Y., del Monte, F., Peluso, R., Zsebo, K., Jeong, D., Park, W.J., Kawase, Y. & Hajjar, R.J. 2007, "Restoration of mechanical and energetic function in failing aortic-banded rat hearts by gene transfer of calcium cycling proteins", *Journal of Molecular and Cellular Cardiology,* vol. 42, no. 4, pp. 852-861.

Sande, J.B., Sjaastad, I., Hoen, I.B., Bokenes, J., Tonnessen, T., Holt, E., Lunde, P.K. & Christensen, G. 2002, "Reduced level of serine(16) phosphorylated phospholamban in the failing rat myocardium: a major contributor to reduced SERCA2 activity", *Cardiovascular research,* vol. 53, no. 2, pp. 382-391.

Schillinger, W., Meyer, M., Kuwajima, G., Mikoshiba, K., Just, H. & Hasenfuss, G. 1996, "Unaltered ryanodine receptor protein levels in ischemic cardiomyopathy", *Molecular and cellular biochemistry,* vol. 160-161, pp. 297-302.

Schmidt, U., Hajjar, R.J., Kim, C.S., Lebeche, D., Doye, A.A. & Gwathmey, J.K. 1999, "Human heart failure: cAMP stimulation of SR Ca(2+)-ATPase activity and phosphorylation level of phospholamban", *The American Journal of Physiology,* vol. 277, no. 2 Pt 2, pp. H474-80.

Sedej, S., Heinzel, F.R., Walther, S., Dybkova, N., Wakula, P., Groborz, J., Gronau, P., Maier, L.S., Vos, M.A., Lai, F.A., Napolitano, C., Priori, S.G., Kockskamper, J. & Pieske, B. 2010, "Na+-dependent SR Ca2+ overload induces arrhythmogenic events in mouse cardiomyocytes with a human CPVT mutation", *Cardiovascular research,* vol. 87, no. 1, pp. 50-59.

Seidler, T., Hasenfuss, G. & Maier, L.S. 2007, "Targeting altered calcium physiology in the heart: translational approaches to excitation, contraction, and transcription", *Physiology (Bethesda, Md.),* vol. 22, pp. 328-334.

Shan, J., Kushnir, A., Betzenhauser, M.J., Reiken, S., Li, J., Lehnart, S.E., Lindegger, N., Mongillo, M., Mohler, P.J. & Marks, A.R. 2010, "Phosphorylation of the ryanodine receptor mediates the cardiac fight or flight response in mice", *The Journal of clinical investigation,* vol. 120, no. 12, pp. 4388-4398.

Simmerman, H.K. & Jones, L.R. 1998, "Phospholamban: protein structure, mechanism of action, and role in cardiac function", *Physiological Reviews,* vol. 78, no. 4, pp. 921-947.

Sussman, M.A., Lim, H.W., Gude, N., Taigen, T., Olson, E.N., Robbins, J., Colbert, M.C., Gualberto, A., Wieczorek, D.F. & Molkentin, J.D. 1998, "Prevention of cardiac hypertrophy in mice by calcineurin inhibition", *Science (New York, N.Y.),* vol. 281, no. 5383, pp. 1690-1693.

Terentyev, D., Kubalova, Z., Valle, G., Nori, A., Vedamoorthyrao, S., Terentyeva, R., Viatchenko-Karpinski, S., Bers, D.M., Williams, S.C., Volpe, P. & Gyorke, S. 2008, "Modulation of SR Ca release by luminal Ca and calsequestrin in cardiac myocytes: effects of CASQ2 mutations linked to sudden cardiac death", *Biophysical journal,* vol. 95, no. 4, pp. 2037-2048.

Thermo Fisher Scientific Inc., a, , *Chemistry of Protein Assays*. Available: http://www.piercenet.com/browse.cfm?fldID=876562B0-5056-8A76-4E0C-B764EAB3A339 [2011, 11.10].

Thermo Fisher Scientific Inc., b, , *Overview of Western Blotting*. Available: http://www.piercenet.com/browse.cfm?fldID=8259A7B6-7DA6-41CF-9D55-AA6C14F31193 [2011, 11/29].

Toischer, K., Rokita, A.G., Unsold, B., Zhu, W., Kararigas, G., Sossalla, S., Reuter, S.P., Becker, A., Teucher, N., Seidler, T., Grebe, C., Preuss, L., Gupta, S.N., Schmidt, K., Lehnart, S.E., Kruger, M., Linke, W.A., Backs, J., Regitz-Zagrosek, V., Schafer, K., Field, L.J., Maier, L.S. & Hasenfuss, G. 2010, "Differential cardiac remodeling in preload versus afterload", *Circulation,* vol. 122, no. 10, pp. 993-1003.

Tsuji, Y., Hojo, M., Voigt, N., El-Armouche, A., Inden, Y., Murohara, T., Dobrev, D., Nattel, S., Kodama, I. & Kamiya, K. 2011, "Ca(2+)-related signaling and protein phosphorylation abnormalities play central roles in a new experimental model of electrical storm", *Circulation,* vol. 123, no. 20, pp. 2192-2203.

van Oort, R.J., McCauley, M.D., Dixit, S.S., Pereira, L., Yang, Y., Respress, J.L., Wang, Q., De Almeida, A.C., Skapura, D.G., Anderson, M.E., Bers, D.M. & Wehrens, X.H. 2010, "Ryanodine receptor phosphorylation by calcium/calmodulin-dependent protein kinase II promotes life-threatening ventricular arrhythmias in mice with heart failure", *Circulation,* vol. 122, no. 25, pp. 2669-2679.

Vandecaetsbeek, I., Raeymaekers, L., Wuytack, F. & Vangheluwe, P. 2009, "Factors controlling the activity of the SERCA2a pump in the normal and failing heart", *BioFactors (Oxford, England),* vol. 35, no. 6, pp. 484-499.

Wehrens, X.H., Lehnart, S.E., Reiken, S., Vest, J.A., Wronska, A. & Marks, A.R. 2006, "Ryanodine receptor/calcium release channel PKA phosphorylation: a critical mediator of heart failure progression", *Proceedings of the National Academy of Sciences of the United States of America,* vol. 103, no. 3, pp. 511-518.

Wehrens, X.H., Lehnart, S.E., Reiken, S.R. & Marks, A.R. 2004, "Ca2+/calmodulin-dependent protein kinase II phosphorylation regulates the cardiac ryanodine receptor", *Circulation research,* vol. 94, no. 6, pp. e61-70.

Williams, R.E., Kass, D.A., Kawagoe, Y., Pak, P., Tunin, R.S., Shah, R., Hwang, A. & Feldman, A.M. 1994, "Endomyocardial gene expression during development of pacing tachycardia-induced heart failure in the dog", *Circulation research,* vol. 75, no. 4, pp. 615-623.

Witcher, D.R., Kovacs, R.J., Schulman, H., Cefali, D.C. & Jones, L.R. 1991, "Unique phosphorylation site on the cardiac ryanodine receptor regulates calcium channel activity", *The Journal of biological chemistry,* vol. 266, no. 17, pp. 11144-11152.

Yeh, Y.H., Wakili, R., Qi, X.Y., Chartier, D., Boknik, P., Kaab, S., Ravens, U., Coutu, P., Dobrev, D. & Nattel, S. 2008, "Calcium-handling abnormalities underlying atrial arrhythmogenesis and contractile dysfunction in dogs with congestive heart failure", *Circulation.Arrhythmia and electrophysiology,* vol. 1, no. 2, pp. 93-102.